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PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2 (PAPP-A2)

FIELD OF THE INVENTION

5 The present invention relates to a novel polypeptide with homology to pregnancy-associated plasma protein-A (PAPP-A). The novel polypeptide according to the invention is denoted PAPP-A2. The invention further relates to novel polynucleotides comprising a nucleic acid sequence encoding such a polypeptide, or a fragment thereof.

10

The invention further relates to methods for using the novel polynucleotides, including fragments thereof as defined herein below, and methods for using the novel polypeptides capable of being produced from such polynucleotides.

15

The invention also relates to expression and purification of recombinant PAPP-A2, and to production of polyclonal and monoclonal antibodies against PAPP-A2, and to the purification of native PAPP-A2 from human tissues or body fluids.

20

In further aspects the invention relates to uses of PAPP-A2 as a marker for pathological states, and as a therapeutic target for drugs that modify the proteolytic activity of PAPP-A2 in pregnant as well as non-pregnant individuals.

BACKGROUND OF THE INVENTION

25

Pregnancy-associated plasma protein-A (PAPP-A)

30

PAPP-A was first isolated in 1974 from pregnancy serum along with other proteins believed to be of placental origin (Lin et al., 1974, Am J Obstet Gynecol 118, 223-36). The concentration in serum reaches about 50 mg/liter at the end of pregnancy (Folkersen et al., 1981, Am J Obstet Gynecol 139, 910-4; Oxvig et al., 1995, J Biol Chem 270, 13645-51). PAPP-A was originally characterized as a high molecular weight homotetramer (Bischof, 1979, Arch Gynecol 227, 315-26; Lin et al., 1974, Am J Obstet Gynecol 118, 223-36; Sinosich, 1990, Electrophoresis 11, 70-8), but it

35 has now been demonstrated that PAPP-A primarily exists in pregnancy serum and

- plasma as a covalent, heterotetrameric 2:2 complex with the proform of eosinophil major basic protein (proMBP), PAPP-A/proMBP (Oxvig et al., 1993, J Biol Chem 268, 12243-6). Only about 1% of PAPP-A in pregnancy serum and plasma is present as a homodimer, as recently demonstrated (Overgaard et al., 2000, J Biol Chem). The existence of the PAPP-A/proMBP complex was revealed, in part, by the isolation of a PAPP-A and a proMBP peptide, linked together by a disulfide bond, from a digest of purified PAPP-A/proMBP (Oxvig et al., 1993, J Biol Chem 268, 12243-6).
- The subunits of the PAPP-A/proMBP complex can be irreversibly separated by reduction of disulfide bonds and denaturation (Oxvig et al., 1993, J Biol Chem 268, 12243-6). In reducing SDS-PAGE, the PAPP-A subunit has an apparent molecular weight of 200 kDa (Oxvig et al., 1994, Biochim Biophys Acta 1201, 415-23), and its 1547-residue sequence is known from cloned cDNA (Kristensen et al., 1994, Biochemistry 33, 1592-8). PAPP-A is synthesized as a pre-pro-protein (preproPAPP-A), including a 80-residue pre-pro-piece (Haaning et al., 1996, Eur J Biochem 237, 159-63). No proteins with global homology to PAPP-A has been reported in the literature, but PAPP-A contains sequence motifs, including an elongated zinc binding motif (HEXXHXXGXXH) at position 482-492 (numbering according to Kristensen et al., 1994, Biochemistry 33, 1592-8). This motif and a structurally important methionine residue, also thought to reside in PAPP-A at position 556, are strictly conserved within the metzincins, a superfamily of zinc peptidases: astacins, adamalysins (or reprotlysins), serralsins and matrixins (matrix metalloproteinases or MMP's) (Bode et al., 1993, FEBS Lett 331, 134-40; Stocker et al., 1995, Protein Sci 4, 823-40).
- The proMBP subunit has a calculated peptide mass of 23 kDa (Barker et al., 1988, J Exp Med 168, 1493-8; McGrogan et al., 1988, J Exp Med 168, 2295-308). In SDS-PAGE, however, proMBP migrates as a smear of 50-90 kDa that is not visible in Coomassie-stained gels (Oxvig et al., 1993, J Biol Chem 268, 12243-6), probably due to its strong and unusual glycosylation (Oxvig et al., 1994, Biochem Mol Biol Int 33, 329-36; Oxvig et al., 1994, Biochim Biophys Acta 1201, 415-23). PAPP-A and proMBP are both produced in the placenta during pregnancy, but mainly in different cell types as shown by *in situ* hybridization (Bonno et al., 1994, Lab Invest 71, 560-6). Analyses by RT-PCR revealed that both PAPP-A and proMBP mRNA are pres-

ent in several reproductive and nonreproductive tissues, although the levels are lower than in the placenta (Overgaard et al., 1999, Biol Reprod 61, 1083-9).

5 Clinical use of PAPP-A

Clinically, depressed serum levels of PAPP-A are increasingly being used as a predictor of Down's syndrome pregnancies (Brambati et al., 1993, Br J Obstet Gynaecol 100, 324-6; Haddow et al., 1998, N Engl J Med 338, 955-61; Wald et al., 1992, Bmj 305, 28; Wald et al., 1999, N Engl J Med 341, 461-7), and it has been shown
10 that PAPP-A serum levels are also depressed in other fetal abnormalities (Biagiotti et al., 1998, Prenat Diagn 18, 907-13; Spencer et al., 2000, Prenat Diagn 20, 411-6; Westergaard et al., 1983, Prenat Diagn 3, 225-32).

Further, the synthesis of PAPP-A in smooth muscle cells of the coronary artery following angioplasty is increased (Bayes-Genis et al., 2000, Arterioscler Thromb Vasc Biol, in press), which is currently being evaluated for potential clinical value. Data
15 show that measurements of proMBP in pregnancy serum also have a diagnostic value (Christiansen et al., 1999, Prenat Diagn 19, 905-10).

20

Proteolytic activity of PAPP-A: Cleavage of IGFBP-4

Only recently, the putative metalloproteinase activity of PAPP-A has been experimentally confirmed (Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53).
25 PAPP-A was partially purified from human fibroblast-conditioned medium (HFCM) and shown to be responsible for the proteolytic activity of HFCM against insulin-like growth factor binding protein (IGFBP)-4. IGFBP's, of which six have been described, are important modulators of IGF-I and -II activity (Fowlkes, 1997, Trends Endocrinol Metab 8, 299-306; Rajaram et al., 1997, Endocr Rev 18, 801-31).
30

IGF-I and -II are essential polypeptides with potent anabolic and mitogenic actions both *in vivo* and *in vitro*. IGF bound to IGFBP-4 cannot interact with its receptor, but bioactive IGF is released once the binding protein is cleaved. Interestingly, cleavage
35 of IGFBP-4 by PAPP-A strictly requires the presence of IGF (Conover et al., 1993, J

Clin Invest 91, 1129-37; Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53). PAPP-A secretion has also been demonstrated from osteoblasts and marrow stromal cells (Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53), from granulosa cells (Conover et al., 1999, J Clin Endocrinol Metab 84, 4742-5), and from vascular smooth muscle cells (Bayes-Genis et al., 2000, Arterioscler Thromb Vasc Biol, in press), all of which have known IGF-dependent IGFBP-4 proteinase activity.

IGFBP-5

Like IGFBP-4, IGFBP-5 cleavage has been widely reported to occur by unidentified proteinases in a number of tissues and conditioned media (Hwa et al., 1999, Endocr Rev 20, 761-87).

SUMMARY OF THE INVENTION

Pregnancy-associated plasma protein-A2

The novel nucleic acid according to the invention has been isolated from human placenta and characterised by means of sequencing analysis. The novel nucleotide sequence encodes a new polypeptide, PAPP-A2.

The amino acid sequence of PAPP-A2 is composed of a 233-residue pre-pro-piece and a 1558-residue mature portion. The mature portion of PAPP-A2 is homologous with the mature portion of PAPP-A (approx. 45 % identity), but the prepro-pieces do not show any similarity between the two proteins. Like PAPP-A, PAPP-A2 contains conserved amino acid stretches that classify it as a putative metalloproteinase of the metzincin superfamily.

PAPP-A2 has been expressed in a mammalian expression system, and it has been demonstrated that PAPP-A2 is an active enzyme. Further, it has been shown that PAPP-A2 cleaves IGFBP-5, Insulin Like Growth Factor Binding Protein 5. In comparison, the cleavage of IGFBP-4 by PAPP-A has previously been demonstrated.

A complementary DNA (cDNA) which encodes the full length form of PAPP-A2 is identified, sequenced and isolated. The cDNA or portions of the cDNA is cloned into

expression vectors for expression in a recombinant host. The cDNA is useful to produce recombinant full-length PAPP-A2 or fragments of PAPP-A2. The cDNA and the recombinant PAPP-A2 protein derived therefrom are useful in the production of antibodies, diagnostic kits, laboratory reagents and assays.

5

The cDNA and the recombinant PAPP-A2 protein may also be used to identify compounds that affect PAPP-A2 function. PAPP-A2 antisense oligonucleotides or antisense mimetics may be clinically useful for reducing the expression of PAPP-A2 protein and thereby antagonizing the effects of PAPP-A. Similarly, the PAPP-A2 coding sequence can be used for gene therapy to introduce PAPP-A2 into target cells thereby enhancing the effects of PAPP-A2.

10

The invention furthermore pertains to PAPP-A2 for use as a therapeutic target for the reduction or elimination of IGFBP-5 proteolytic activity in a cell.

15

Other features and advantages of the invention will be apparent from the following drawings and description hereof, from the following detailed description, and from the claims.

20

DEFINITIONS

25

As used herein, PAPP-A2 refers to an isolated PAPP-A2 polypeptide having the amino acid sequence listed in Fig. 1 (SEQ ID NO:2), or a variant thereof as defined herein. The PAPP-A2 according to the invention, or a variant thereof, may be produced by recombinant DNA technology, or the PAPP-A2 may be naturally occurring.

A PAPP-A2 encoding nucleotide sequence refers to an isolated nucleic acid having the sequence listed in Fig. 1 (SEQ ID NO:1), or a variant thereof as defined herein.

30

"Active" refers to those forms of PAPP-A2 which retain the biological and/or immunological activities of any naturally occurring PAPP-A2.

35

"Naturally occurring PAPP-A2" refers to PAPP-A2 produced by human cells that have not been genetically engineered and specifically contemplates various PAPP-A2s arising from post-translational modifications of the polypeptide including but not

limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation, acylation, or complex formation, covalent or noncovalent, with other polypeptides.

5 An "isolated polypeptide" is a protein, or a variant or fragment thereof, which constitutes 90% or more of the protein contents of a given preparation as evaluated by standard methods known in the art of protein chemistry.

10 "Derivative" refers to polypeptides derived from naturally occurring PAPP-A2 by chemical modifications such as ubiquitination, labeling (e.g., with radionuclides, various enzymes, etc.), pegylation (derivatization with polyethylene glycol), or by insertion (or substitution by chemical synthesis) of amino acids (amino acids) such as ornithine, which do not normally occur in human proteins.

15 "Recombinant variant" refers to any polypeptide differing from naturally occurring PAPP-A2 by amino acid insertions, deletions, and substitutions, created using recombinant-DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as proteolytic activity or cell adhesion, may be found e.g. by comparing parts of the sequence of PAPP-A2 with structurally similar proteins (e.g. other metzincin family
20 proteinases), with locally homologous proteins of known disulfide structure, or by secondary structure predictions.

25 Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as, but not limited to, the replacement of a leucine with an isoleucine or valine, replacement of an aspartate with a glutamate, or replacement with a threonine with a serine, i.e., conservative amino acid replacements. Further examples and definitions falling within the meaning of the term "substitutions" as applied herein are provided in the detailed description of the invention herein below.

30

Amino acid "insertions" or "deletions" are typically in the range of from about about 1 amino acid to about 50 amino acids, such as from about 1 amino acid to about 20 amino acids, for example from about 1 amino acid to about 20 amino acids, such as from about 1 amino acid to about 10 amino acids. The variation allowed may be ex-
35 perimentally determined by systematically making insertions, deletions, or substitu-

tions of amino acids in a PAPP-A2 molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

5 Where desired, a "signal or leader sequence" can direct the polypeptide (full length PAPP-A2, or portions of the PAPP-A2 polypeptide) through the membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

10 A polypeptide "fragment", "portion", or "segment" is a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, typically at least about 9 to 13 amino acids, such as at least about 17 or more amino acids in various embodiments. It may also be a longer stretch of residues up to intact PAPP-A2 in length. To be active, any PAPP-A2 polypeptide or PAPP-A2 polypeptide fragment
15 must have sufficient length to display biologic and/or immunologic activity on their own, or when conjugated to a carrier protein such as keyhole limpet-hemocyanin.

An "oligonucleotide" or polynucleotide "fragment", "portion", or "segment" is a stretch of the PAPP-A2 encoding sequence which is useful in the expression of PAPP-A2
20 polypeptide fragments. It may also be a stretch of nucleotide residues capable of being used in a polymerase chain reaction (PCR) or a hybridization procedure, typically for amplifying or revealing related parts of mRNA or DNA molecules. In particular, one or both oligonucleotide probes will comprise sequence that is identical or complementary to a portion of PAPP-A2 where there is little or no identity or complementarity with any known or prior art molecule. For this purpose, such oligonucleotide probes will generally comprise between about 10 nucleotides and 50 nucleotides,
25 and preferably between about 15 nucleotides and about 30 nucleotides.

"Animal" as used herein may be defined to include human, domestic or agricultural
30 (cats, dogs, cows, sheep, etc) or test species (mouse, rat, rabbit, etc).

"Recombinant" may also refer to a polynucleotide which encodes PAPP-A2 and is prepared using recombinant DNA techniques. The DNAs which encode PAPP-A2 may also include allelic or recombinant variants and mutants thereof.

"Nucleic acid probes" are prepared based on the cDNA sequences which encode PAPP-A2 provided by the present invention. Nucleic acid probes comprise portions of the sequence having fewer nucleotides than about 6 kb, usually fewer than about 1 kb. After appropriate testing to eliminate false positives, these probes may be used to determine whether mRNAs encoding PAPP-A2 are present in a cell or tissue or to isolate similar nucleic acid sequences from chromosomal DNA extracted from such cells or tissues as described in (Walsh et al., 1992, PCR Methods Appl 1, 241-50). Probes may be derived from naturally occurring or recombinant single- or double-stranded nucleic acids or be chemically synthesized. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes of the present invention, their preparation and/or labeling are elaborated in (Sambrook et al., 1989); or (Ausubel et al., 1989).

Alternatively, recombinant variants encoding these PAPP-A2 or similar polypeptides may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations may also be introduced to modify the properties of the polypeptide, including but not limited to activity, interchain affinities, or polypeptide degradation or turnover rate. One example involves inserting a stop codon into the nucleotide sequence to limit the size of PAPP-A2 so as to provide a molecule of smaller molecular weight.

"Expression vectors" are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

The term "antibody" as used herein includes both polyclonal and monoclonal antibodies, as well as fragments thereof, such as, Fv, Fab and F(ab)2 fragments that are capable of binding antigen or hapten. It includes conventional murine monoclonal antibodies as well as human antibodies, and humanized forms of non-human antibodies, and it also includes 'antibodies' isolated from phage antibody libraries.

"Ribozymes" are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of PAPP-A2 RNA sequences. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between fifteen (15) and twenty (20) ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

DETAILED DESCRIPTION OF THE INVENTION

Isolation of a nucleotide sequence encoding PAPP-A2

The present invention in one aspect relates to a novel cDNA sequence encoding a protein with global homology to pregnancy-associated plasma protein-A (PAPP-A). This protein has been denoted PAPP-A2. The complete nucleotide sequence of PAPP-A2 has been obtained from mRNA isolated from human placenta (Example 1). The complete nucleotide sequence (SEQ ID NO:1) and translated amino acid sequence (SEQ ID NO:2) of PAPP-A2 are both shown in Figure 1.

Homology of PAPP-A2 with PAPP-A is evident upon alignment of the two amino acid sequences as shown in Figure 3. PAPP-A2 and PAPP-A share approximately 45% of their amino acid residues. Sequence motifs known to be important for the function of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8; Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53; Overgaard et al., 2000, J Biol Chem) are also found in PAPP-A2. Principally, PAPP-A2 contains an elongated zinc binding motif (HEXXHXXGXXH, amino acids shown by one letter code) at position 733-743 (Figure 2). This motif and a structurally important methionine residue, are

strictly conserved within the metzincins, a superfamily of zinc peptidases (Bode et al., 1993, FEBS Lett 331, 134-40; Stocker et al., 1995, Protein Sci 4, 823-40).

Like PAPP-A, PAPP-A2 is synthesized as a prepro-protein. PreproPAPP-A2 has 1791 amino acids (Figure 1). There is no homology between the prepro-portions of PAPP-A and PAPP-A2. Further, the prepro-portions of the two proteins differ significantly in length. The PAPP-A2 prepro-peptide has 233 residues (Figure 3); the PAPP-A prepro-peptide has 80 residues (Haaning et al., 1996, Eur J Biochem 237, 159-63).

Uses of the nucleotide sequence encoding PAPP-A2

The nucleotide sequence encoding PAPP-A2 (or its complement) have numerous applications in techniques known to those skilled in the art of molecular biology.

These techniques include use as hybridization probes, use in the construction of oligomers for PCR, use in the recombinant production of PAPP-A2 or fragments hereof, and use in generation of anti-sense DNA or RNA, their chemical analogs (e.g. PNA or LNA) and the like. Uses of nucleotides encoding PAPP-A2 disclosed herein are exemplary of known techniques and are not intended to limit their use in any technique known to a person of ordinary skill in the art. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, e.g., the triplet genetic code, specific base pair interactions, etc.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of PAPP-A2-encoding nucleotide sequences, some bearing minimal homology to the nucleotide sequence of any known and naturally occurring gene may be produced. The invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring PAPP-A2, and all such variations are to be considered as being specifically disclosed.

Although the nucleotide sequences which encode PAPP-A2 and/or its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring PAPP-A2 under stringent conditions, it may be advantageous to produce nucleotide sequences encoding PAPP-A2 or its derivatives possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding PAPP-A2 and/or its derivatives without altering the encoded amino acid sequence include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

Nucleotide sequences encoding PAPP-A2 may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (Sambrook et al., 1989). Useful nucleotide sequences for joining to PAPP-A2 include an assortment of cloning vectors, e.g., plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art. Vectors of interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for the host cell.

Another aspect of the subject invention is to provide for PAPP-A2-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences encoding PAPP-A2. Such probes may also be used for the detection of similar PAPP-A2 encoding sequences and should preferably contain at least 50% of the nucleotides from the conserved region or active site. The hybridization probes of the subject invention may be derived from the nucleotide sequences of the SEQ ID NO:1 or from genomic sequences including promoters, enhancer elements and/or possible introns of the respective naturally occurring PAPP-A2. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as ^{32}P or ^{35}S , or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

PCR as described (U.S. Pat. Nos 4,683,195; and 4,965,188) provides additional uses for oligonucleotides based upon the nucleotide sequence which encodes PAPP-A2. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both and comprise a discrete nucleotide sequence for diagnostic use or a degenerate pool of possible sequences for identification of closely related genomic sequences.

Other means of producing specific hybridization probes for PAPP-A2 DNAs include the cloning of nucleic acid sequences encoding PAPP-A2 or PAPP-A2 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides.

It is possible to produce a DNA sequence, or portions thereof, encoding PAPP-A2 and their derivatives entirely by synthetic chemistry, after which the gene can be inserted into any of the many available DNA vectors using reagents, vectors and cells that are known in the art at the time of the filing of this application. Moreover, synthetic chemistry may be used to introduce mutations into the PAPP-A2 sequences or any portion thereof.

The nucleotide sequence can be used in an assay to detect disease associated with abnormal levels of expression of PAPP-A2. The nucleotide sequence can be labeled by methods known in the art and added to a fluid or tissue sample from a patient under hybridizing conditions. After an incubation period, the sample is washed with a compatible fluid which optionally contains a dye (or other label requiring a developer) if the nucleotide has been labeled with an enzyme. After the compatible fluid is rinsed off, the dye is quantitated and compared with a standard. Alternatively, levels of PAPP-A2 mRNA can be measured by micro array techniques using immobilized probes. Expression in samples can also be evaluated by (semi-quantitative) RT-PCR. Expression in samples can alternatively be evaluated by techniques based on hybridization. For example, *in situ* hybridization can be used to detect PAPP-A2 mRNA. This technique has the advantage that it locates the cells that synthesize the mRNA, but also is less sensitive than RT-PCR.

Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of PAPP-A2. Antisense techniques are known in the art and may be applied herein. Both antisense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

The invention also relates to unknown PAPP-A2 genes isolated from other species and alleles of the PAPP-A2 gene, in which PAPP-A2 orthologues or homologues exists. A bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a radioactively labeled fragment of the human PAPP-A2 clone described herein. Alternatively the human PAPP-A2 sequence can be used to design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the PAPP-A2 sequences. The PCR fragment may be used to isolate a full length PAPP-A2 clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., (Ausubel et al., 1989; Sambrook et al., 1989).

Expression of recombinant PAPP-A2

In order to express a biologically active proteinase, the nucleotide sequence coding for the protein, or a functional equivalent, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. For example, recombinant

protein can be used for immunization to obtain antibodies, as a laboratory reagent, and in diagnostic kits.

5 More specifically, methods which are well known to those skilled in the art can be used to construct expression vectors containing the PAPP-A2 sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See e.g., the techniques described in (Ausubel et al., 1989; Sambrook et al., 1989).

10

Further, expression vectors containing fragments of the PAPP-A2 encoding sequence may also be constructed. In particular, this may be relevant for the use of portions of the PAPP-A2 polypeptide as an antigen for immunization. In addition, the coding sequence of PAPP-A2 or fragments hereof may be cloned in frame with a
15 coding nucleotide sequence present in the vector to result in a fusion protein or a "tagged" PAPP-A2 protein. For example, such a fusion protein may be composed of PAPP-A2 and GST, and such tag may be a c-myc tag (for detection) and/or a histidine tag (for purification).

20

A variety of host-expression vector systems may be utilized to express the PAPP-A2 coding sequence or fragments hereof. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the PAPP-A2 coding sequence; yeast transformed with recombinant yeast expression vectors containing
25 the PAPP-A2 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the PAPP-A2 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the PAPP-A2 coding
30 sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus, human tumor cells) including cell lines engineered to contain multiple copies of the PAPP-A2 DNA either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage lambda, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the CMV promoter, the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the PAPP-A2 DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce PAPP-A2 protein. Identification of PAPP-A2 expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-PAPP-A2 antibodies, and the presence of host cell-associated PAPP-A2 activity.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the PAPP-A2 expressed. For example, when large quantities of PAPP-A2 are to be produced, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector pUR278 (Ruther and Muller-Hill, 1983, Embo J 2, 1791-4), in which the PAPP-A2 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from

lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety. In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, see (Ausubel et al., 1989; Bitter et al., 1987, Methods Enzymol 153, 516-44; Rosenfeld, 1999, Methods Enzymol 306, 154-69).

In cases where plant expression vectors are used, the expression of the PAPP-A2 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV may be used (Gmunder and Kohli, 1989, Mol Gen Genet 220, 95-101); alternatively, plant promoters such as the small subunit of RUBISCO (Broglie et al., 1984, Science 224, 838-43).

An alternative expression system which could be used to express PAPP-A2 is an insect system. In one such system, Baculovirus is used as a vector to express foreign genes. The virus then grows in the insect cells. The PAPP-A2 coding sequence may be cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control of a Baculovirus promoter. These recombinant viruses are then used to infect insect cells in which the inserted gene is expressed. For example, see (Smith et al., 1983, Mol Cell Biol 3, 2156-65).

A variety of mammalian expression vectors may be used to express recombinant PAPP-A2 in mammalian cells. Commercially-available mammalian expression vectors which may be suitable for recombinant PAPP-A2 expression, include but are not limited to, pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 37110), pcDNA3.1 and its derivatives (Stratagene). Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1, COS-1, COS-7, CHO-K1, 3T3, NIH3T3, HeLa, C127I, BS-C-1, MRC-5, and 293. Further, in mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the PAPP-A2 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric

gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing PAPP-A2 in infected hosts. See for example (Logan and Shenk, 1984, Proc Natl Acad Sci U S A 81, 3655-9). Alternatively, the vaccinia 7.5K promoter may be used. See for example (Mackett et al., 1982, Proc Natl Acad Sci U S A 79, 7415-9).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express PAPP-A2 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with PAPP-A2 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

Some applications of the recombinant PAPP-A2 may require the protein to be in purified or partially purified form. Recombinantly expressed PAPP-A2 or fragments of the PAPP-A2 polypeptide can be isolated by liquid chromatography. Various methods of protein purification well known in the art include those described in for example (Scopes, 1987). Alternatively, recombinant PAPP-A2 fusion proteins or 'tagged' PAPP-A2 may be purified by affinity chromatography. Further, antibodies raised against PAPP-A2 may be used for purification by immunoaffinity chromatography.

Recombinant variant of PAPP-A2 may be produced by site directed mutagenesis. In some applications of PAPP-A2 such variants may be preferred due to for example increased protein stability, or changes in activity.

Production and uses of antibodies against PAPP-A2

The recombinant protein may be used to generate antibodies. Monospecific antibodies to PAPP-A2 can be purified from mammalian antisera containing antibodies

reactive against PAPP-A2 or can be prepared as monoclonal antibodies reactive with PAPP-A2 using standard techniques.

5 Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for PAPP-A2. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the PAPP-A2, as described above. PAPP-A2 specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits or
10 mice being preferred, with an appropriate concentration of PAPP-A2 either with or without an immune adjuvant. For example, antibodies specific against PAPP-A2 can be used for the purification of native and recombinant PAPP-A2, as a laboratory reagent, and in antibody based diagnostic kits.

15 Monoclonal antibodies (mAb) reactive with PAPP-A2 can be prepared by conventional methods, such as by immunizing inbred mice with PAPP-A2. The mice are immunized with about 0.1 mg to about 10 mg, preferably about 1 mg, of PAPP-A2 in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant. Freund's complete adjuvant is preferred. The mice receive an initial immuniza-
20 tion on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of PAPP-A2 in a buffer solution such as phosphate buffered saline (PBS) by the intravenous (IV) route. Lymphocytes from antibody-positive mice are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are
25 produced by mixing the splenic lymphocytes with an appropriate fusion partner under conditions which will allow the formation of stable hybridomas. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18,
30 and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using PAPP-A2 as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are then cloned. For details, see
35 (Peters and Baumgarten, 1992).

In vitro production of anti-PAPP-A2 is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

5 Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique (Crowther, 1995).

10 The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in (Clackson et al., 1991, Nature 352, 624-8; Marks et al., 1991, J Mol Biol 222, 581-97), for example. Identified phage antibodies can be produced by expression in bacteria.

15 Methods such as those described above may be used to produce monospecific antibodies-specific for PAPP-A2 polypeptide fragments or full-length nascent PAPP-A2 polypeptide.

20 PAPP-A2 antibody affinity columns can be made by adding the antibodies to a gel support, such as Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M
25 glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing PAPP-A2 or PAPP-A2 fragments are slowly passed through the column. The column is then washed, and the protein is eluted. The purified PAPP-A2 protein is then dialyzed against phosphate buffered
30 saline.

Native PAPP-A2 from sources such as human plasma or serum, tissue extracts, or media from nontransfected cell lines (that endogenously secrete PAPP-A2) may also be purified by use of an antibody affinity column.

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Using polyclonal or monoclonal antibodies against PAPP-A2 a number of assays may be constructed for measurement of PAPP-A2 antigen in body fluids or tissue and cell extracts. Kits based on antibodies may be used for diagnostic purposes. The assays include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent assay (ELISA) techniques, and radioimmunoassay (RIA) techniques.

For example, in one such ELISA, a sandwich assay can be constructed where antigen present in an sample is caught by immobilized polyclonal anti(PAPP-A2). Detection is then performed by the use of one or more monoclonal PAPP-A2 antibodies and peroxidase conjugated anti(murine IgG). In another assay, antigen present in an sample is caught by immobilized polyclonal anti(PAPP-A2), and detected using biotinylated polyclonal anti(PAPP-A2). For further examples and details, see (Crowther, 1995). Assays can be calibrated using purified PAPP-A2 to construct a standard curve by serial dilution. The concentration of PAPP-A2 in solution in a purified form can be accurately measured by amino acid analysis (Sottrup-Jensen, 1993, Biochem Mol Biol Int 30, 789-94).

Polyclonal antibodies may be used to inhibit the biological activity of PAPP-A2. Specifically, in analogy with the inhibition of the IGFBP-4 proteolytic activity of PAPP-A by polyclonal PAPP-A antibodies (Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53), anti(PAPP-A2) may be used to inhibit the proteolytic activity of PAPP-A2. Certain monoclonal antibodies may also be inhibitory towards the activity of PAPP-A2. Such monoclonal antibodies are likely to recognize an epitope in close proximity to the active site of PAPP-A2, but the inhibitory activity may also be based on binding to epitopes other than those close to the active site. Inhibitory monoclonal antibodies can be obtained by immunization with PAPP-A2, PAPP-A2 fragments, with peptides derived from PAPP-A2.

Inhibitory (monoclonal) antibodies may have therapeutic value in conditions of pathologies in which it may be desirable to decrease the activity of PAPP-A2.

Activity of PAPP-A2

Like PAPP-A, PAPP-A2 contains conserved amino acid stretches that classify it as a putative metalloproteinase of the metzincin superfamily (Stocker et al., 1995, Protein Sci 4, 823-40). It has been experimentally verified that PAPP-A2 does exhibit proteolytic activity by demonstrating its cleavage of insulin-like growth factor binding protein (IGFBP)-5 (Example 6.7).

In general, proteolytic activity of PAPP-A2 against potential protein substrates may be evaluated by the incubation of purified or partially purified PAPP-A2 with the potential substrate under a variety of experimental conditions (such as for example temperature, buffer composition, ionic strength, and pH). Enzymatic activity of PAPP-A2 against the protein in question can be evaluated by SDS-PAGE (in which degradation or release of well defined proteolytic fragment(s) will be evident), or by high-pressure liquid chromatographic detection of released peptide(s). By means of such procedures, other substrate targets of PAPP-A2 may be identified. Incubation with a variant of PAPP-A2 where, for example, a residue in the active site has been substituted to obtain an inactive enzyme, serves as a proper negative control.

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that can be cleaved by PAPP-A2. Identification of such peptides may be accomplished by screening a peptide library with recombinant soluble PAPP-A2. Methods for expression and purification of the enzyme are described above and may be used to express recombinant full length PAPP-A2 or fragments, analogs, or derivatives thereof depending on the functional domains of interest. For further details, see (Meldal, 1998, Methods Mol Biol 87, 65-74; Meldal, 1998, Methods Mol Biol 87, 51-7). Alternatively, peptide substrates may be derived from identified protein substrates of PAPP-A2.

Alternatively, phage display of peptide libraries may be used to identify peptides that can be cleaved by PAPP-A2 (Matthews and Wells, 1993, Science 260, 1113-7).

Peptides that function as PAPP-A2 substrates may function in assays for the detection of PAPP-A2 proteolytic activity in body fluids or tissue and cell extracts. Sub-

strate peptides may be derivatized to function in an assay based on quenched-fluorescence (Meldal, 1998, Methods Mol Biol 87, 65-74). Kits based on such, or other, techniques may be used for diagnostic purposes in pathologies where measurement of PAPP-A2 activity is relevant.

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Identification of agents that modify the activity of PAPP-A2

An assay for the detection of PAPP-A2 proteolytic activity, as described above, provides a method for the identification of molecules that modify the activity of PAPP-A2. Such molecules may be, for example, peptides, derivatized peptides, hydroxamic acid derivatized peptides, small organic molecules, or antibodies.

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The screening of peptide libraries can be used to discover pharmaceutical agents that act to modulate and/or inhibit the biological activity of PAPP-A2. Methods for expression and purification of the enzyme are described above and may be used to express recombinant full length PAPP-A2 or fragments, analogs, or derivatives thereof depending on the functional domains of interest. Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to modulate and/or inhibit PAPP-A2 activity by binding to the active site or other sites of PAPP-A2. For example, see (Meldal, 1998, Methods Mol Biol 87, 75-82).

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Similarly, combinatorial chemistry may be used to identify low molecular weight organic molecules that affect the activity of PAPP-A2.

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Measurement of complexes of PAPP-A or PAPP-A2

PAPP-A primarily exists in pregnancy serum as a disulfide bound 2:2 complex with the proform of eosinophil major basic protein (proMBP), PAPP-A/proMBP (see section 2.2). The level of complex of PAPP-A2 and proMBP (PAPP-A2/proMBP), of complex of PAPP-A2 and PAPP-A (PAPP-A2/PAPP-A), or of complex of PAPP-A/PAPP-A2 with proMBP (PAPP-A2/PAPP-A/proMBP) can be measured using specific monoclonal antibodies towards the individual components. For example, measuring PAPP-A/proMBP by sandwich ELISA using a PAPP-A mAb for catching and a

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proMBP mAb for detection may be used alternatively to predict Down's syndrome pregnancies, or other clinical conditions (see section 2.3).

Use of PAPP-A2 to generate natural proteolytic fragments

PAPP-A2 may be used to generate natural fragments of proteins that are specifically cleaved by PAPP-A2. As in the case of IGFBP-5 (see Examples 6.7 and 6.9), such fragments may have biological effects different from intact IGFBP-5. Fragments can be purified by standard chromatography after cleavage with purified PAPP-A2 (see Example 6.9).

Design of fragments of PAPP-A2 for expression

Because all cysteine residues found in mature PAPP-A are also found in mature PAPP-A2 (see Figure 3), the pattern of disulfide bonds can be assumed to be the same for PAPP-A2 for those common cysteine residues. Therefore, knowledge of the disulfide structure of the PAPP-A subunit (see Figure 8) can be used to rationally design fragments of PAPP-A2 in which pairing of all cysteine residues is possible. Putative domain boundaries of PAPP-A2 can be defined based on the disulfide structure shown in Figure 8. Those domains can be expressed separately or in combination. In the event that a domain contains a cysteine residue known to form an inter-chain disulfide bridge to another PAPP-A subunit or to proMBP (see Figure 8), it may be required that this cysteine is mutated to for example a serine or an alanine residue

Thus, possible boundary regions are *between* Cys-403 and Cys-499, between Cys-828 and Cys-881, between Cys-1048 and Cys-1115, between Cys-1390 and Cys-1396, between Cys-1459 and Cys-1464, between Cys-1521 and Cys-1525, between Cys-1590 and Cys-1595, between Cys-1646 and Cys-1653, and between Cys-1729 and Cys-1733 (numbering of preproPAPP-A2, as in Figure 1 and 3).

Pharmaceutical Compositions

Identification of PAPP-A2 as the IGFBP-5 protease provides methods for affecting growth and differentiation *in vivo* by using PAPP-A2 as a therapeutic target.

Inhibitors of PAPP-A2 is believed to decrease the amount of bioavailable IGF-I and IGF-II. For example, inhibition of PAPP-A2 activity can be useful in disorders such as restenosis, atherosclerosis, and fibrosis. Activators, or agents that increase the activity of PAPP-A2, is believed to increase the amount of bioavailable IGF-I and IGF-II.

Agents that alter PAPP-A2 activity or that alter adherence of PAPP-A2 to cell surfaces can be incorporated into pharmaceutical compositions. Such agents may be incorporated together with agents that alter PAPP-A activity or that alter adherence of PAPP-A to cell surfaces. A combination of PAPP-A2 specific agents and PAPP-A specific agents may be more effective than traditional agents directed against PAPP-A. There is also provided a method of treatment comprising the step of administering to an individual in need thereof a combination of PAPP-A2 specific agents and PAPP-A specific agents in pharmaceutically effective amounts.

As an example, an antibody such as anti-PAPP-A2 polyclonal or monoclonal, can be formulated into a pharmaceutical composition by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compounds and compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions in aqueous physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal drops, or aerosols. Compositions for other routes of administration may be prepared as desired using standard methods.

Formulations for parenteral administration may contain as common excipients (i.e., pharmaceutically acceptable carriers) sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for controlling the release of a compound of the invention in vivo. Other suitable parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain excipients such as lactose, if desired. Inhalation formulations may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or they may be oily

solutions for administration in the form of nasal drops. If desired, the compounds can be formulated as gels to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration

5 Medical Devices

The invention also features a medical device for placement in a patient (e.g., an implant) that includes an agent that inhibits or activates PAPP-A2 protease activity. Suitable agents are readily identified using the methods described herein. The device can be impregnated with the agent or can be coated with the agent. Non-limiting examples of inhibitors include an antibody such as anti-PAPP-A2 polyclonal or monoclonal, or a metalloprotease inhibitor such as 1,10-phenanthroline.

IGFBP-5 protease activity of PAPP-A2 is potently inhibited by 1,10-phenanthroline, but is not inhibited by tissue inhibitors of matrix metalloproteases (TIMP'S). Other inhibitors include small molecules such as derivatives of hydroxamic acid. Anti-PAPP-A2 polyclonal IgG may also inhibit IGF-dependent - or IGF-independent - IGFBP-5 specific PAPP-A2 protease activity in HFCM in a dose-dependent manner.

In addition, polypeptides (i.e., any chain of amino acids, regardless of length or post-translational modification), including modified polypeptides, can function as inhibitors. Any inhibitor of the IGFBP-5 protease activity of PAPP-A2 can be used for coating or impregnating a medical device according to the invention. Modified polypeptides include amino acid substitutions, deletions, or insertions in the amino acid sequence as compared with a corresponding wild-type sequence, as well as chemical modifications. Although protease-resistant IGFBP-5 is not an inhibitor per se of the IGFBP-5 protease activity of PAPP-A2, similar results are expected when it is used for coating or impregnating a medical device.

As an example, coating or impregnating the medical device with a PAPP-A2 inhibitor, optionally in combination with a PAPP-A inhibitor, can help prevent the development of restenosis following balloon angioplasty, or can prevent a further increase in size of an atherosclerotic plaque. Coronary angioplasty with stent placement is currently the leading therapeutic approach for coronary atherosclerosis. An important goal of angioplasty of coronary artery disease is to prevent both acute and chronic

complications. Modern procedures are quite successful in eliminating immediate problems. Unfortunately, restenosis still occurs in 20-30% of stented patients. No known pharmacological intervention is available to prevent the restenosis.

- 5 Without being bound by a particular mechanism, it is thought that an increase in IGFBP-5 protease expression by coronary smooth muscle cells precedes neointimal formation in response to angioplasty in humans.

- 10 For example, enhanced PAPP-A2 activity can be useful for wound healing, fractures, osteoporosis, or ovulation. Osteoporosis or other conditions of bone loss may benefit from increased bone formation and decreased bone resorption. Agents that enhance PAPP-A2 activity can be, for example, a modified IGF, i.e., an IGF analog.

- 15 Analogs include IGF polypeptides containing amino acid insertions, deletions or substitutions, as well as chemical modifications. Amino acid substitutions can include conservative and non-conservative amino acid substitutions. Conservative amino acid substitutions replace an amino acid with an amino acid of the same class, whereas non-conservative amino acid substitutions replace an amino acid
- 20 with an amino acid of a different class. Non-conservative substitutions result in a change in the hydrophobicity of the polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions can make a substantial change in the charge of the polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions
- 25 include a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid. Amino acid insertions, deletions and substitutions can be made using random mutagenesis, site-directed mutagenesis, or other recombinant techniques known in the art.

- 30 The medical device can be, for example, bone plates or bone screws that are used to stabilize bones, or a stent, which typically is used within the body to restore or maintain the patency of a body lumen. Blood vessels, for example, can become obstructed due to an atherosclerotic plaque that restricts the passage of blood. A stent typically has a tubular structure defining an inner channel that accommodates
- 35 flow within the body lumen. The outer walls of the stent engage the inner walls of

the body lumen. Positioning of a stent within an affected area can help prevent further occlusion of the body lumen and permit continued flow. A stent typically is deployed by percutaneous insertion of a catheter or guide wire that carries the stent. The stent ordinarily has an expandable structure. Upon delivery to the desired site, the stent can be expanded with a balloon mounted on the catheter. Alternatively, the stent may have a biased or elastic structure that is held within a sheath or other restraint in a compressed state. The stent expands voluntarily when the restraint is removed. In either case, the walls of the stent expand to engage the inner wall of the body lumen, and generally fix the stent in a desired position.

STATEMENTS OF INVENTION

In a first aspect the present invention relates to a purified polynucleotide selected from the group consisting of

- i) a polynucleotide comprising nucleotides 1 to 5376 of SEQ ID NO:1, corresponding to the coding sequence of PAPP-A2, as deposited with DSMZ under accession number DSM 13783; and
- ii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and
- iii) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment
 - a) has a proteolytic activity specific for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5), or a derivative thereof, or any other substrate; and/or
 - b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide; and

5 iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), said polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2, or a fragment thereof, wherein said fragment

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a) has a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or

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b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

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c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide; and

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v) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iii) and (iv),

and the complementary strand of such a polynucleotide.

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A polynucleotide as used herein shall denote any naturally occurring polynucleotide having any naturally occurring backbone structure, as well as nucleotides known in the art as LNA (locked nucleic acid) and PNA (peptide nucleic acid).

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In preferred embodiments the purified polynucleotide comprises the coding sequence of PAPP-A2, nucleotides 1 to 5376, as shown in SEQ ID NO:1, or a nucleotide sequence encoding the amino acid sequence as shown in SEQ ID NO:2.

In another preferred embodiment the polynucleotide comprises a nucleotide sequence encoding a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:2, wherein said fragment

- 5 a) has a proteolytic activity specific for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5), or a derivative thereof, or any other substrate; and/or
- 10 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
- 15 c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide

There is also provided a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide according to the invention.

20 Stringent conditions as used herein shall denote stringency as normally applied in connection with Southern blotting and hybridization as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100
25 μg/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and a washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

30 The DNA sequences are used in a variety of ways. They may be used as probes for identifying homologs of uHase (e.g., homologs of huHase). Mammalian homologs have substantial sequence similarity to one another, i.e. at least 75%, usually at least 90%, more usually at least 95% sequence identity. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger se-

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quence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul et al. 1990 J Mol Biol 215:403-10.

Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50.degree. C. and 10.times.SSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subjected to washing at 55.degree. C. in 1.times.SSC. Sequence identity may be determined by hybridization under high stringency conditions, for example, at 50.degree. C. or higher and 0.1.times.SSC (9 mM saline/0.9 mM sodium citrate). By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes may be any species, e.g. Primate species, particularly human; rodents, such as rats and mice, canines, felines, bovine, opines, equine, yeast, Drosophila, Caenorhabditis, etc.

In a further embodiment there is provided a polynucleotide comprising a nucleotide sequence which is degenerate to a polynucleotide capable of hybridising to SEQ ID NO:1, or a fragment thereof.

Degeneracy as used herein is defined in terms of the activity or functionality associated with the polypeptide expressed from said degenerate polynucleotide, said polynucleotide is either i) comprising a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or ii) recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or iii) competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide.

In a further embodiment there is provided a polynucleotide comprising the complementary strand of a polynucleotide according to the invention.

The polynucleotide according to the invention may be operably linked to a further polynucleotide comprising nucleic acid residues corresponding to the 3' untranslated

region of PAPP-A2, or a fragment thereof. As used herein the 3' untranslated region comprises nucleic acid residues 5377 to 8527 of SEQ ID NO:1.

5 There is also provided a recombinant DNA molecule in the form of an expression vector comprising an expression signal operably linked to a polynucleotide according to the invention.

10 In a further embodiment there is provided a host organism transfected or transformed with the polynucleotide according to the invention, or the vector according to the invention. The host organism is preferably a mammalian organism such as e.g. a mammalian cell line. However, a microbial eukaryote such as yeast or fungi may also be used, as may a microbial prokaryote such as Bacillus or E. coli. The person skilled in the art will know how to select expression signals, including leader sequences and/or signal peptides suitable for expression in a given cell. The person
15 skilled in the art will also know how to determine the level of expression in a given cell by using standard molecular biology techniques.

In a further aspect the invention relates to an isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:2, or a fragment
20 thereof, wherein said fragment

- a) has a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or
- 25 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
- c) competes with a polypeptide having the amino acid sequence as shown in
30 SEQ ID NO:2 for binding to a cell surface receptor with an affinity for said polypeptide.

In one preferred embodiment of the invention there is also provided variants of SEQ ID NO:2, and variants of fragments thereof. Variants are determined on the basis of
35 their degree of identity or their homology with a predetermined amino acid se-

quence, said predetermined amino acid sequence being SEQ ID NO:2, or, when the variant is a fragment, a fragment of SEQ ID NO:2.

Accordingly, variants preferably have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity, such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 % sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity with the predetermined sequence.

Variants are also determined based on a predetermined number of conservative amino acid substitutions as defined herein below. Conservative amino acid substitution as used herein relates to the substitution of one amino acid (within a predetermined group of amino acids) for another amino acid (within the same group), wherein the amino acids exhibit similar or substantially similar characteristics.

Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within the groups of amino acids indicated herein below:

- i) Amino acids having polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys,)
- ii) Amino acids having non-polar side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp, Pro, and Met)
- iii) Amino acids having aliphatic side chains (Gly, Ala Val, Leu, Ile)
- iv) Amino acids having cyclic side chains (Phe, Tyr, Trp, His, Pro)
- v) Amino acids having aromatic side chains (Phe, Tyr, Trp)

- vi) Amino acids having acidic side chains (Asp, Glu)
- vii) Amino acids having basic side chains (Lys, Arg, His)
- 5 viii) Amino acids having amide side chains (Asn, Gln)
- ix) Amino acids having hydroxy side chains (Ser, Thr)
- x) Amino acids having sulphur-containing side chains (Cys, Met),
- 10 xi) Neutral, weakly hydrophobic amino acids (Pro, Ala, Gly, Ser, Thr)
- xii) Hydrophilic, acidic amino acids (Gln, Asn, Glu, Asp), and
- 15 xiii) Hydrophobic amino acids (Leu, Ile, Val)

Accordingly, a variant or a fragment thereof according to the invention may comprise, within the same variant of the sequence or fragments thereof, or among different variants of the sequence or fragments thereof, at least one substitution,

20 such as a plurality of substitutions introduced independently of one another.

It is clear from the above outline that the same variant or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

25 The addition or deletion of an amino acid may be an addition or deletion of from 2 to 10 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 10 to 100 amino acids, addition of

30 100 to 150 amino acids, addition of 150-250 amino acids, are also comprised within the present invention.

The polypeptide fragments according to the present invention, including any functional equivalents thereof, may in one embodiment comprise less than 250

35 amino acid residues, such as less than 240 amino acid residues, for example less

than 225 amino acid residues, such as less than 200 amino acid residues, for example less than 180 amino acid residues, such as less than 160 amino acid residues, for example less than 150 amino acid residues, such as less than 140 amino acid residues, for example less than 130 amino acid residues, such as less than 120 amino acid residues, for example less than 110 amino acid residues, such as less than 100 amino acid residues, for example less than 90 amino acid residues, such as less than 85 amino acid residues, for example less than 80 amino acid residues, such as less than 75 amino acid residues, for example less than 70 amino acid residues, such as less than 65 amino acid residues, for example less than 60 amino acid residues, such as less than 55 amino acid residues, for example less than 50 amino acid residues.

"Functional equivalency" as used in the present invention is according to one preferred embodiment established by means of reference to the corresponding functionality of a predetermined fragment of the sequence. More specifically, functional equivalency is to be understood as the ability of a polypeptide fragment to exert IGFBP-5 specific protease activity and/or to be recognised by an antibody capable of recognising PAPP-A2 and/or to compete with PAPP-A2 for binding to a receptor having affinity for PAPP-A2.

Functional equivalents or variants of PAPP-A2 will be understood to exhibit amino acid sequences gradually differing from the preferred predetermined PAPP-A2 sequence, as the number and scope of insertions, deletions and substitutions including conservative substitutions increases. This difference is measured as a reduction in homology between the preferred predetermined sequence and the fragment or functional equivalent.

All fragments or functional equivalents of SEQ ID NO:2 are included within the scope of this invention, regardless of the degree of homology that they show to a preferred predetermined sequence of PAPP-A2 as reported herein. The reason for this is that some regions of PAPP-A2 are most likely readily mutable, or capable of being completely deleted, without any significant effect on the binding activity of the resulting fragment.

- A functional variant obtained by substitution may well exhibit some form or degree of native PAPP-A2 activity, and yet be less homologous, if residues containing functionally similar amino acid side chains are substituted. Functionally similar in this respect refers to dominant characteristics of the side chains such as hydrophobic, basic, neutral or acidic, or the presence or absence of steric bulk. Accordingly, in one embodiment of the invention, the degree of identity is not a principal measure of a fragment being a variant or functional equivalent of a preferred predetermined fragment according to the present invention.
- The homology between amino acid sequences may be calculated using well known algorithms such as BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, or BLOSUM 90.
- Fragments sharing at least some homology with fragments of SEQ ID NO:2 are to be considered as falling within the scope of the present invention when they are at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with said fragments of SEQ ID NO:2. According to one embodiment of the invention the homology percentages refer to identity percentages.
- Additional factors that may be taken into consideration when determining functional equivalence according to the meaning used herein are i) the ability of antisera to detect a PAPP-A2 fragment according to the present invention, or ii) the ability of the functionally equivalent PAPP-A2 fragment to compete with PAPP-A2 in a binding assay. One method of determining a sequence of immunogenically active amino acids within a known amino acid sequence has been described by Geysen in US 5,595,915 and is incorporated herein by reference.

A further suitably adaptable method for determining structure and function relationships of peptide fragments is described by US 6,013,478, which is herein

incorporated by reference. Also, methods of assaying the binding of an amino acid sequence to a receptor moiety are known to the skilled artisan.

Conservative substitutions may be introduced in any position of a preferred predetermined fragment of SEQ ID NO:2, and it may also be desirable to introduce non-conservative substitutions in any one or more positions.

A non-conservative substitution leading to the formation of a functionally equivalent fragment of PAPP-A2 would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Variants obtained by substitution of amino acids may in one preferred embodiment be made based upon the hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition to the variants described herein, sterically similar variants may be formulated to mimic the key portions of the variant structure and that such compounds may also be used in the same manner as the variants of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

In a further embodiment the present invention relates to functional comprising substituted amino acids having hydrophilic or hydrophobic indices that are within ± 2.5 , for example within ± 2.3 , such as within ± 2.1 , for example within ± 2.0 , such as within ± 1.8 , for example within ± 1.6 , such as within ± 1.5 , for example within ± 1.4 , such as within ± 1.3 for example within ± 1.2 , such as within ± 1.1 , for example within ± 1.0 , such as within ± 0.9 , for example within ± 0.8 , such as within ± 0.7 , for example within ± 0.6 , such as within ± 0.5 , for example within ± 0.4 , such as within ± 0.3 , for example within ± 0.25 , such as within ± 0.2 of the value of the amino acid it has substituted.

The importance of the hydrophilic and hydrophobic amino acid indices in conferring interactive biologic function on a protein is well understood in the art (Kyte & Doolittle, 1982 and Hopp, U.S. Pat. No. 4,554,101, each incorporated herein by reference).

The amino acid hydrophobic index values as used herein are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5) (Kyte & Doolittle, 1982).

The amino acid hydrophilicity values are: arginine (+3.0); lysine (+3.0); aspartate (+3.0, ± 0.1); glutamate (+3.0, ± 0.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5, ± 0.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4) (U.S. 4,554,101).

In addition to the peptidyl compounds described herein, sterically similar compounds may be formulated to mimic the key portions of the peptide structure and that such compounds may also be used in the same manner as the peptides of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. For example, esterification and other alkylations may be employed to modify the amino terminus of, e.g., a di-arginine peptide backbone, to

mimic a tetra peptide structure. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

5 Peptides with N-terminal alkylations and C-terminal esterifications are also encompassed within the present invention. Functional equivalents also comprise glycosylated and covalent or aggregative conjugates formed with the same or other PAPP-A2 fragments and/or PAPP-A2 molecules, including dimers or unrelated chemical moieties. Such functional equivalents are prepared by linkage of functionalities to groups which are found in fragment including at any one or both of the N- and C-termini, by means known in the art.

10 Functional equivalents may thus comprise fragments conjugated to aliphatic or acyl esters or amides of the carboxyl terminus, alkylamines or residues containing carboxyl side chains, e.g., conjugates to alkylamines at aspartic acid residues; O-acyl derivatives of hydroxyl group-containing residues and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g. conjugates with fMet-Leu-Phe or immunogenic proteins. Derivatives of the acyl groups are selected from the group of alkyl-moieties (including C3 to C10 normal alkyl), thereby forming alkanoyl species, and carbocyclic or heterocyclic compounds, thereby forming aroyl species. The reactive groups preferably are difunctional compounds known per se for use in cross-linking proteins to insoluble matrices through reactive side groups.

25 Covalent or aggregative functional equivalents and derivatives thereof are useful as reagents in immunoassays or for affinity purification procedures. For example, a fragment of PAPP-A2 according to the present invention may be insolubilized by covalent bonding to cyanogen bromide-activated Sepharose by methods known per se or adsorbed to polyolefin surfaces, either with or without glutaraldehyde cross-linking, for use in an assay or purification of anti-PAPP-A2 antibodies or cell surface receptors. Fragments may also be labelled with a detectable group, e.g., radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates or conjugated to another fluorescent moiety for use in e.g. diagnostic assays.

30 Mutagenesis of a preferred predetermined fragment of PAPP-A2 can be conducted by making amino acid insertions, usually on the order of about from 1 to 10 amino

acid residues, preferably from about 1 to 5 amino acid residues, or deletions of from about from 1 to 10 residues, such as from about 2 to 5 residues.

5 In one embodiment the fragment of PAPP-A2 is synthesised by automated synthesis. Any of the commercially available solid-phase techniques may be employed, such as the Merrifield solid phase synthesis method, in which amino acids are sequentially added to a growing amino acid chain. (See Merrifield, J. Am. Chem. Soc. 85:2149-2146, 1963).

10 Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied Biosystems, Inc. of Foster City, Calif., and may generally be operated according to the manufacturer's instructions. Solid phase synthesis will enable the incorporation of desirable amino acid substitutions into any fragment of PAPP-A2 according to the present invention. It will be understood that substitutions,
15 deletions, insertions or any subcombination thereof may be combined to arrive at a final sequence of a functional equivalent. Insertions shall be understood to include amino-terminal and/or carboxyl-terminal fusions, e.g. with a hydrophobic or immunogenic protein or a carrier such as any polypeptide or scaffold structure capable as serving as a carrier.

20 Oligomers including dimers including homodimers and heterodimers of fragments of PAPP-A2 according to the invention are also provided and fall under the scope of the invention. PAPP-A2 functional equivalents and variants can be produced as homodimers or heterodimers with other amino acid sequences or with native PAPP-A2
25 sequences. Heterodimers include dimers containing immunoreactive PAPP-A2 fragments as well as PAPP-A2 fragments that need not have or exert any biological activity.

PAPP-A2 fragments according to the invention may be synthesised both in vitro and
30 in vivo. Method for in vitro synthesis are well known, and methods being suitable or suitably adaptable to the synthesis in vivo of PAPP-A2 are also described in the prior art. When synthesized in vivo, a host cell is transformed with vectors containing DNA encoding PAPP-A2 or a fragment thereof. A vector is defined as a replicable nucleic acid construct. Vectors are used to mediate expression of PAPP-A2. An expression
35 vector is a replicable DNA construct in which a nucleic acid sequence en-

coding the predetermined PAPP-A2 fragment, or any functional equivalent thereof that can be expressed in vivo, is operably linked to suitable control sequences capable of effecting the expression of the fragment or equivalent in a suitable host. Such control sequences are well known in the art.

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Cultures of cells derived from multicellular organisms represent preferred host cells. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI38, BHK, COS-7, 293 and MDCK

10 cell lines. Preferred host cells are eukaryotic cells known to synthesize endogenous PAPP-A2. Cultures of such host cells may be isolated and used as a source of the fragment, or used in therapeutic methods of treatment, including therapeutic methods aimed at promoting or inhibiting a growth state, or diagnostic methods carried out on the human or animal body.

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In particular embodiments the present invention relates to a polypeptide fragment according to the invention, wherein the PAPP-A2 fragment comprises or essentially consists of amino acid residues 234 to 1791 corresponding to the mature part of PAPP-A2, including any processing variants thereof.

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Processing variants are variants resulting from alternative processing events, possibly processing events catalysed by any protease including, but not limited to, a signal peptidase and a furin. One putative cleavage site is located after position 233 is described herein below in detail. Another putative cleavage site is located after the motif RQRR (position 196 - 199 in the amino acid sequence of PAPP-A2). Process-

25 ing variants shall be understood to comprise variants arising from processing in vivo when PAPP-A2 is expressed in human or animal tissue, sera or body fluids.

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Mature PAPP-A2 amino acids sequences essentially consisting of the mature sequence designated in SEQ ID NO:2 (amino acid residues 234 to 1791) shall be understood in one embodiment to comprise this part of the sequence lacking between 1 to about 10 N-terminal amino acids or C-terminal amino acids, preferably 1 to 10 N-terminal amino acids, such as 2 to 8 N-terminal acids, for example 3 to 6 N-terminal amino acids.

Also included in the definition of essentially consisting of as used herein shall be the mature sequence designated in SEQ ID NO:2 (amino acid residues 234 to 1791) having in addition thereto an additional 1 to about 10 N-terminal amino acids or C-terminal amino acids, preferably 1 to 10 N-terminal amino acids, such as 2 to 8 N-terminal acids, for example 3 to 6 N-terminal amino acids. This definition of essentially consisting of shall also apply in other aspects and is not restricted to being used in connection with a particular part of PAPP-A2. The definition shall also apply to other processes PAPP-A2 polypeptides including polypeptides arising from alternative processing in tissue, sera or body fluids other than the ones from where the processed PAPP-A2 has originally been isolated.

Additionally preferred fragments comprise or essentially consists of amino acid residues 1 to 233 corresponding to the prepro part of PAPP-A2, of amino acid residues 23 to 233 corresponding to the pro part of PAPP-A2, of amino acid residues 1 to 22 corresponding to the signal peptide or leader sequence of PAPP-A2, and to such sequences operably linked to the mature part of PAPP-A2 corresponding to amino acid residues 234 to 1791 of SEQ ID NO:2.

There is also provided recombinant PAPP-A2 polypeptide, or a fragment thereof, wherein preferably the polypeptide is free of human proteins, or other proteins natively associated with said polypeptide.

In a further aspect there is provided a composition comprising i) a polynucleotide according to the invention, and/or ii) a vector according to the invention, and/or iii) a host organism according to the invention, and/or iv) a polypeptide according to the invention, in combination with a physiologically acceptable carrier.

In yet another aspect there is provided a pharmaceutical composition comprising i) a polynucleotide according to the invention, and/or ii) a vector according to the invention, and/or iii) a host organism according to the invention, and/or iv) a polypeptide according to the invention, in combination with a pharmaceutically acceptable carrier.

The invention further pertains to a method for producing an antibody with specificity for a PAPP-A2 polypeptide according to the invention, or a fragment thereof, said method comprising the steps of

- 5 i) providing a host organism,
- ii) immunizing the host organism with the polypeptide according to claim 10,
 and
- 10 iii) obtaining said antibody.

There is also provided monoclonal antibodies and polyclonal antibodies having specific binding affinity for a PAPP-A2 polypeptide according to the invention, or a fragment thereof. The antibody is preferably a monoclonal.

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In a further aspect there is provided a method for producing a PAPP-A2 polypeptide according to the invention, said method comprising the steps of

- i) providing a suitable host organism, preferably a mammalian cell,
- 20 ii) transfecting or transforming the host organism provided in step i) with a
 polynucleotide according to the invention, or a vector according to the in-
 vention,
- 25 iii) culturing the host organism obtained in step ii) under conditions suitable
 for expression of the polypeptide encoded by the polynucleotide or the
 vector; and optionally
- iv) isolating from the host organism the polypeptide resulting from recombi-
30 nant expression by the host organism.

In a still further aspect of the invention there is provided a method for inhibiting and/or reducing the expression of PAPP-A2 in a cell by means of anti-sense technology, said method comprising the steps of

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- i) providing an anti-sense polynucleotide according to the invention,
- ii) transfecting or transforming a cell capable of expressing PAPP-A2 with said anti-sense polynucleotide provided in step i),
- 5 iii) culturing the cell obtained in step ii) under conditions suitable for hybridization of the polynucleotide provided in step i) to a complementary polynucleotide in said cell involved in the expression of PAPP-A2, and
- 10 iv) inhibiting and/or reducing the expression of PAPP-A2 in said cell.

The antisense polynucleotide and the complementary polynucleotide may be co-expressed from distinct polynucleotide molecules or they may be expressed from the same molecule. As an alternative to hybridization, the method may include the use of reverse transcriptase PCR technology (rt PCT technology).

In yet another aspect of the invention there is provided a method for detecting PAPP-A2, or measuring the level of PAPP-A2, in a biological sample obtained from an individual, said method comprising the steps of

- 20 i) obtaining a biological sample from said individual,
- ii) detecting PAPP-A2 in said sample by detecting
 - 25 a) a PAPP-A2 polypeptide, or a fragment thereof, and/or
 - b) a polynucleotide in the form of mRNA originating from PAPP-A2 expression, and/or
 - 30 c) PAPP-A2 specific protease activity, preferably IGFBP-5 protease activity, or proteolytic activity directed against a derivative of IGFBP-5..

The method may comprise the further step of comparing the PAPP-A2 or the level of PAPP-A2 detected in step ii) with a predetermined value selected from the group consisting of

- a) a predetermined amount and/or concentration of PAPP-A2; and/or
- b) a predetermined amount and/or concentration of PAPP-A2 mRNA;
and/or
- c) a predetermined PAPP-A2 specific protease activity.

The predetermined value in one embodiment will be indicative of a normal physiological condition of said individual.

The biological sample is preferably selected from the group consisting of blood, urine, pleural fluid, oral washings, tissue biopsies, and follicular fluid.

When the level of PAPP-A2 is measured as an amount of PAPP-A2 protein, the PAPP-A2 protein is preferably measured by immunochemical analysis wherein PAPP-A2 protein is detected by at least one monoclonal antibody. PAPP-A2 protein may also be detected in a complex comprising at least one additional component, preferably a polypeptide such as, but not limited to, pro-MBP (pro-Major-Basic Protein). PAPP-A2 may also be detected as a PAPP-A2 monomer or as a PAPP-A2 dimer.

Further aspects of the invention relates to a method of diagnosing a clinical condition in an individual, said method comprising the steps of

- i) performing a method for detecting PAPP-A2 or measuring the level of PAPP-A2, and
- ii) diagnosing the clinical condition.

The clinical condition is preferably a fetal abnormality such as, but not limited to, a fetal abnormality selected from the group consisting of Trisomy 21, Trisomy 18, Trisomy 13, and Open Spina Bifida.

Additional fetal abnormalities capable of being diagnosed according to the invention is ectopic pregnancy, open spina bifida, neural tube defects, ventral wall defects, Edwards Syndrome, Patau's Syndrome, Turner Syndrome, Monosomy X or Klinefelter's Syndrome.

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In another aspect the clinical condition is an altered growth state selected from the group consisting of a growth promoting state and a growth inhibiting state, including, but not limited to, restenosis, atherosclerosis, wound healing, fibrosis, myocardial infarction, osteoporosis, rheumatoid arthritis, multiple myeloma, or cancer.

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In a yet further aspect of the invention there is provided a method for detecting expression of a polynucleotide according to the invention in a biological sample, said method comprising the steps of

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i) providing a biological sample putatively containing a polynucleotide according to the invention, and

ii) contacting the biological sample with a polynucleotide comprising a strand that is i) complementary to the polynucleotide according to the invention and ii) capable of hybridizing thereto, and

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iii) allowing hybridization to occur, and

iv) detecting the hybridization complex obtained in step iii),

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wherein the presence of the hybridization complex is indicative of the expression in the biological sample of the polynucleotide according to the invention, or a fragment thereof.

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In a still further aspect of the invention there is provided a method for identifying an agent inhibiting the protease activity of PAPP-A2, said method comprising the steps of

- i) incubating a) the polypeptide according to the invention, or a fragment thereof, and b) a predetermined substrate for said polypeptide or fragment, and c) a putative inhibitory agent, and
- 5 ii) determinining if proteolysis of said substrate is inhibited.

The substrate preferably comprises comprises a polypeptide that may be an internally quenched fluorescent peptide. One preferred substrate comprises or essentially consists of IGFBP-5, or a fragment thereof.

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The invention also pertains to an inhibitory agent obtainable according to such a method for identifying an agent inhibiting the protease activity of PAPP-A2.

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There is also provided the use of such provided inhibitory agents in the manufacture of a medicament for treating a clinical condition in an individual in need of such treatment.

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In a still further aspect the invention pertains to a method for identifying an agent capable of enhancing the protease activity of PAPP-A2, said method comprising the steps of

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- i) incubating a) the polypeptide according to the invention, or a fragment thereof, and b) a predetermined substrate for said polypeptide, and c) a putative enhancer agent, and
- ii) determinining if proteolysis of said substrate is enhanced.

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The substrate preferably comprises a polypeptide including an internally quenched fluorescent peptide. IGFBP-5, or a fragment thereof, is particularly preferred as a substrate.

There is also provided an enhancing agent obtainable according to the method for identifying an agent capable of enhancing the protease activity of PAPP-A2, and the invention also pertains to the use of such enhancing agents in the manufacture of a

medicament for treating a clinical condition in an individual in need of such treatment.

5 In yet another aspect there is provided a method of treatment by therapy of an individual, said method comprising the step of administrating to said individual i) a pharmaceutical composition according to the invention, and/or ii) the inhibitory agent according to the invention, and/or the enhancing agent according to the invention.

10 In a still further aspect there is provided a method for purification of PAPP-A2 or complexes of PAPP-A2 with other proteins, said method comprising the steps of

- i) providing a polyclonal or monoclonal antibody with specific binding affinity for a polypeptide according to the invention, or a fragment thereof, and
- 15 ii) purifying PAPP-A2, or a fragment thereof, by means of affinity chromatography.

20 It is understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cDNA sequence (in 5'→3' orientation) corresponding to the mRNA that encodes preproPAPP-A2. Only the coding part of the sequence and the
 30 terminal stop codon (*) is shown and is numbered 1-5376. The translated polypeptide sequence of preproPAPP-A2 is also shown. The signal peptide cleavage site was predicted using SignalP V2.0 to be after the alanine residue encoded by nt. 64-66 ((Nielsen et al., 1997, Protein Eng 10, 1-6), WWW prediction server is located at <http://genome.cbs.dtu.dk/>). The signal peptide of preproPAPP-A2 (nt. 1-66, 22 residues)
 35 is shown in bold. The nucleotide sequence of this figure represents nt. 1 to

5376 of SEQ ID NO:1. The protein sequence of this figure is illustrated as SEQ ID NO:2.

Figure 2 is a schematic drawing of the relationship between PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8), and sequence stretches contained within two genomic clones with homology to the N-terminal end (hom-N, coding portion of accession number AL031734) and the C-terminal end (hom-C, coding portion of accession number AL031290) of PAPP-A, when translated into amino acid sequence. This figure also illustrates the method by which a cDNA sequence with homology to the midregion of PAPP-A was obtained. Hom-N, hom-C, and the midregion together encodes the complete sequence of a novel protein, PAPP-A2, which is a homolog of PAPP-A. The midregion was obtained by PCR using specifically primed (primer RT-N-mid), reversed transcribed human placental mRNA as the template, and primers PR-mid5 and PR-mid3 for the PCR (Table 1). To obtain a cDNA construct encoding the full-length PAPP-A2, cDNA clones corresponding to the genomic clones hom-N and hom-C were also obtained using cDNA synthesized with specifically primed placental mRNA as the template (primers not shown, see Table 1). This required identification of a signal peptide stretch (in hom-N) and a stop codon (at the 3' end of hom-C), as detailed in the main text. All primers used are shown in Table 1. Note: The relative positions of the sequences depicted here are in accordance with the experiments performed, but the figure is not accurately drawn to scale.

Figure 3 shows the amino acid sequence of preproPAPP-A2 (SEQ ID NO:2) aligned with preproPAPP-A. The deduced amino acid sequence of preproPAPP-A2 (PA2) was aligned with the sequence of preproPAPP-A (PA) ((Haaning et al., 1996, Eur J Biochem 237, 159-63), AAC50543) using CLUSTAL W (Thompson et al., 1994, Nucleic Acids Res 22, 4673-80). Because the prepro-portion of PAPP-A did not show significant identity with the corresponding region of PAPP-A2, the alignment was manually adjusted to emphasize difference in length of pro-peptides. Arrows indicate the N-termini of the mature proteins as found earlier for PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) (Glu-81), and here for PAPP-A2 (Ser-234). Putative signal peptides, strongly predicted using SignalP V2.0 (Nielsen et al., 1997, Protein Eng 10, 1-6) are shown with lower case letters. The pro-portion of PAPP-A2 contains one other candidate initiation codon corresponding to Met-168, but no signal peptide was predicted following this residue using SignalP. The sequence motifs of

PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) are also found in PAPP-A2: The catalytic zinc binding motif and residues of the putative Met-turn are underlined and bolded in both sequences. Lin-notch motifs (LNR1-3) and short consensus repeats (SCR-1-5) are boxed. Cysteine residues are shaded. All cysteines of mature PAPP-A are also found in PAPP-A2. In addition, the secreted form of PAPP-A2 has four cysteine residues (Cys-343, Cys-533, Cys-618, and Cys-1268) with no counterpart in PAPP-A.

Figure 4 shows PAPP-A2 by Western blotting and Coomassie staining. Medium from transfected 293T cells was Western blotted using monoclonal anti-*c-myc*. Lane 1, cells transfected with empty vector; lane 2, cells transfected with cDNA encoding wild-type PAPP-A2 C-terminally tagged with the *c-myc* peptide (pPA2-mH), non-reduced; lane 3, cells transfected with or cDNA encoding PAPP-A2 with an inactivating E734Q mutation (pPA2-KO-mH), non-reduced; lane 4, as lane 2, but reduced. Recombinant PAPP-A2 was purified by nickel affinity chromatography from serum free medium of cells transfected with pPA2-KO-mH, to eliminate possible autocatalysis (lane 5, reduced).

Figure 5 shows the activity of PAPP-A2 against IGFBP-1-6. Medium from 293T cells transfected with empty vector (-), or cDNA encoding PAPP-A2 (pPA2) (+) was incubated with each of the six IGFBPs (BP1-BP6), and the activity was assessed by ligand blotting using radiolabeled IGF-II. Complete cleavage of IGFBP-5 is evident from the absence of a signal in the BP5+ lane. Partial degradation of IGFBP-3 is also evident.

Figure 6 shows proteolytic activity of PAPP-A2 against IGFBP-5. Medium from 293T cells transfected with empty vector (lane 1), cDNA encoding PAPP-A2 with an inactivating E734Q mutation (pPA2-KO) (lane 2), or cDNA encoding wild-type PAPP-A2 (pPA2) (lanes 3-6) was incubated with C-terminally *c-myc* tagged rIGFBP-5. Proteolytic activity was assessed by Western blotting using anti-*c-myc*. 'i' denotes intact rIGFBP-5; 'c' denotes the detectable C-terminal *c-myc* tagged cleavage product. In the absence of inhibitors, wild-type PAPP-A2 degraded all rIGFBP-5 (lane 3). The PAPP-A2 activity was abolished by 10 mM phenantroline (lane 4) and 5 mM EDTA (lane 5), but not affected by 100 μ M 3,4-DCI (lane 6). Coomassie-stained SDS-PAGE of purified rIGFBP-5 is shown before (lane 7) and after (lane 8) digestion with

purified PAPP-A2. A Western blot of the same digest, using anti-c-myc, is also shown (lane 9). Sequence analysis revealed that PAPP-A2 cleaves IGFBP-5 at one site, between Ser-142 and Lys-143.

5 Figure 7 shows the cDNA sequence of the PAPP-A2 mRNA coding region directly followed by the sequence of the 3'UTR. The sequence of the 3'UTR was obtained as detailed in Example 6.3 The first 5376 nucleotides of this sequence (nt. 1 - 5376) represents the coding sequence as illustrated in Figure 1 and SEQ ID NO:1 (nt. 1 - 5376). Nucleotides 5377 - 8527 of this sequence corresponds to the 3'UTR of the
10 PAPP-A2 mRNA as illustrated in SEQ ID NO:3 (nt. 5377 - 8527).

Figure 8 shows the disulfide structure of the PAPP-A subunit in the PAPP-A/proMBP complex (upper bar). Cysteine containing peptides originating from the PAPP-A/proMBP complex were isolated by degrading PAPP-A/proMBP complex with pro-
15 teinases and cyanogen bromide followed by standard HPLC. Peptides were identified by amino acid analysis, N-terminal sequence analysis, and by mass spectrometry (Overgaard, M. T., Oxvig, C., unpublished). Disulfide bonds are shown by thin lines. Two cysteine residues form inter-chain disulfide bridges to proMBP, and one forms an inter-chain bridge to PAPP-A causing it to be a dimer (as indicated).
20 Asterisks mark a cysteine residue to which no partner has been found. The cysteine residues present in mature PAPP-A is also present in mature PAPP-A2 (see Figure 3). It is reasonable to assume that the disulfide pairing of PAPP-A2 is the same. Thus, this information is valuable in determination of boundary regions for expression of isolated domains (fragments) of PAPP-A2. The gene structure of PAPP-A is
25 also show (lower bar). Exon/intron boundaries are based on comparison of PAPP-A cDNA (AN X68280) with genomic sequences (ANs AB020878, AL353141, and AL137024). The central bar shows putative domains of PAPP-A based on information of the upper and lower bars.

30

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EXAMPLES

6.1. Identification of a nucleotide sequence encoding PAPP-A2

- 5 Accession numbers (ANs) given in this text refer to sequences deposited in GenBank or other biological sequence databases. ANs are used interchangeably with the protein or nucleotide sequences deposited under the given AN.

10 Searching public nucleotide databases for DNA sequences with homology to PAPP-A ((Kristensen et al., 1994, Biochemistry 33, 1592-8), AN CAA48341) when translated into polypeptide sequence revealed two genomic clones with the ANs AL031734 and AL031290. Both originate from the human chromosome 1 (1q24). The search was performed against the "nr" collection of databases using the program tblastn at <http://www.ncbi.nlm.nih.gov/BLAST/> with default settings. In this example, PAPP-A is numbered with the N-terminal Glu as residue 1, as in (Kristensen et al., 1994, Biochemistry 33, 1592-8). In the deposited sequence record (AN X68280) this Glu is residue 5.

20 The sequence reported in AL031734 contains 168835 base pairs. Two noncontiguous sequence stretches (nt. 103432-103566, and 140846-141919) of the total sequence together aligned with residues 16-59, and 59-413 of the PAPP-A polypeptide sequence when translated. The sequence reported in AL031290 contains 121780 base pairs. Four noncontiguous sequence stretches (nt. 10209-10358, 11752-11901, 20531-20463, and 60536-60652) of the total sequence together aligned with residues 1313-1362, 1376-1425, 1457-1479, and 1470-1506 of the PAPP-A polypeptide sequence when translated. The sequence stretches between the coding regions of both of the genomic sequences represent noncoding genomic DNA (introns) or coding regions that do not align.

30 Based on these findings, we hypothesized the existence of a novel protein, PAPP-A2, with homology to PAPP-A. It was then established the complete coding sequence of the regions of PAPP-A2 that were partially covered by the two genomic sequences reported in AL031734 and AL031290. We denote those contiguous sequences hom-N and hom-C, respectively (Figure 2). But first, we established the existence of a coding cDNA sequence that also showed homology to PAPP-A, and

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that connected the sequence of hom-N and hom-C (Figure 2). All essential primers used are described in Table 1. The entire cDNA sequence encoding the 1791-residue preproPAPP-A2 is shown in Figure 1. Standard cloning techniques were used, and all DNA constructs were analyzed by sequencing. The methodology used is described below. The name PAPP-A2 is used for the protein encoded by this DNA sequence.

Cloning of a contiguous coding cDNA stretch corresponding to the midregion between hom-N and hom-C: To obtain the midregion (Figure 2), cDNA was synthesized using human placental mRNA as a template and a primer, RT-N-mid, derived from AL031290 (Table 1, Figure 2). This cDNA was used as a template in a PCR to obtain a cDNA corresponding to the midregion of the hypothesized PAPP-A2. PCR primers were PR-mid5 and PR-mid3 (Table 1, Figure 2). The coding sequence of the midregion obtained corresponds to residues 665-1572 of Figure 3 (SEQ ID NO:1), a total of 908 amino acids.

TABLE 1. Locations of primers used for reverse transcription or PCR. The primers are listed in the order of their use.

	<u>NAME</u>	<u>SOURCE^a</u>	<u>Nt. NUMBERS^b</u>	<u>SEQUENCE^c</u>
	RT-N-mid:	AL031290	10262-10281, (4770-4789)	GCTCACACACCACAGGAATG*
	PR-mid5:	AL031734	141874-141894, (1947-1967)	GGCTGATGTGCGCAAGACCTG
	PR-mid3:	AL031290	10208-10229, (4716-4737)	GCATTGTATCTTCAGGAGCTTG*
	PR-N5:	AL031734	102606-102628, (-)	GAAGTTGACTTCTGGTTCTGTAG
	PR-N3:	-	-, (2380-2400)	CCCTGGGAAGCGAGTGAAGCC*
	RT-C:	AL031290	62982-63006, (-)	GCATTTCTTATAAGATCCTTCATGC*
	PR-C5:	-	-, (4180-4201)	GACAGCTGTCCGTCATTGCTGC
	PR-C3:	AL031290	62876-62897, (-)	CTTACTGCCTCTGAGGCAGTGG*

^aAccession numbers of the relevant genomic clones are given. Primers PR-N3 and PR-C5 were located in the sequence connecting hom-N and hom-C, and are therefore not represented in the databases.

^bNucleotide numbers refer to the numbering of the sequences as reported in the file with the relevant accession number. In parentheses are given the corresponding numbers of SEQ ID NO:1 (Figure 1), except for primers PR-N5, RT-C and PR-C3, not within this sequence.

5

^cSequences are actual primer sequences (orientation 5'-to-3'). Sequences marked with an asterisk are complementary to the database sequences or the sequence given in Figure 1.

10 Cloning of a contiguous coding cDNA stretch corresponding to the N-terminal end of PAPP-A2 (hom-N): Manual inspection of the genomic sequence AL031734 revealed that the open reading frame of the sequence stretch corresponding to PAPP-A residues 16-59 continued further in the 5' direction: Nt. 102646-103566 encodes a polypeptide sequence of 307 residues that starts with a methionine residue. Based on
15 this finding, the cDNA used to obtain the midregion (placental mRNA primed with RT-N-mid, as detailed above) was used as a template in a PCR to obtain the contiguous cDNA of hom-N. PCR primers were: PR-N5 and PR-N3 (Table 1, Figure 2).

20 Cloning of a contiguous coding cDNA stretch corresponding to the C-terminal end of PAPP-A2 (hom-C): Searching available databases (using the program blastn at <http://www.ncbi.nlm.nih.gov/BLAST/> with default settings) for human EST sequences matching the genomic sequence of AL031290 revealed an EST sequence overlapping with some of the coding regions of AL031290 already defined by the stretch nt. 60536-60652 (cf. above). Nt. 62790-62995 of AL031290 also matched
25 the sequence of the human EST sequence AA368081 originating from placenta. When translated into polypeptide sequence, this EST sequence showed homology to the C-terminal end of PAPP-A. Further, a stop codon was present within the coding sequence corresponding to amino acid 1537 of PAPP-A. That is, PAPP-A2 does not extend C-terminally beyond PAPP-A when the two sequences are aligned.
30 Based on this, cDNA was synthesized using human placental mRNA as a template and a primer originating from AL031290 (Table 1). This cDNA was used as a template in a PCR to obtain the contiguous cDNA of hom-C using PCR primers PR-C5 and PR-C3 (Table 1, Figure 2).

35 All PCRs were carried out with *Pfu* polymerase (Stratagene). The three overlapping PAPP-A2 cDNA fragments (hom-N, the novel midregion, and hom-C) were all

cloned into the vector pCR-BluntII-TOPO (Invitrogen). Several clones were sequenced in both orientations. The constructs are referred to as p2N, p2Mid, and p2C, respectively. The entire nucleotide sequence encoding PAPP-A2 is shown in Figure 1 (and SEQ ID NO:1).

5

6.2. Analyses of the nucleotide and amino acid sequence of PAPP-A2

Of the 1547 residues of mature PAPP-A, 708 residues (45.8%) are identical in pre-proPAPP-A2. There is no significant degree of identity between the prepro portion of PAPP-A and the remaining (N-terminal) portion of PAPP-A2 (Figure 3). In this example, PAPP-A is numbered according to ((Haaning et al., 1996, Eur J Biochem 237, 159-63), AAC50543).

The sequence motifs recognized in PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) are also present PAPP-A2: An elongated zinc binding consensus sequence, three lin-notch repeats (LNR1-3), and five short consensus repeats (SCR1-5) (Figure 3). Further, all 82 cysteine residues of PAPP-A are conserved between the two proteins, and an additional 4 cysteines are present in the PAPP-A2 polypeptide sequence.

20

6.3. Identification of human EST sequences originating from the PAPP-A2 mRNA

A cluster of EST sequences matching the genomic sequence of AL031290 were identified around nt 64000-66000 of AL031290, starting approximately 1.2 kb from the end of the PAPP-A2 encoding sequence. The existence of mRNA connecting the coding region of PAPP-A2 and this cluster was verified in a PCR using primers from AL031290 (5'-GGAAAGAGCAGAGTTCACCCAT-3', nt. 64900-64879 of AL031290) and the PAPP-A2 encoding sequence (5'-CCGTCTTAGTCCACTGCATCC-3', nt. 20499-20519 of AL031290, nt 5171-5191 of AF311940), and oligo-dT primed placental cDNA as a template (Overgaard et al., 1999, Biol Reprod 61, 1083-9). As expected, the size of the resulting product was 2.2 kb, further demonstrating the existence of a PAPP-A2 mRNA with a 3'UTR of about 3 kb. The distribution among tissues is shown in Table 2.

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TABLE 2. Expression of PAPP-A2 mRNA in human tissues evaluated by available EST sequences^a.

	Tissue of origin	Number of ESTs found
5	Human placenta	38
	Pregnant uterus	21
	Fetal liver/spleen	11
	Kidney	5
10	Retina/Fetal retina	3
	Corneal stroma	2
	Fetal heart	2
	Gessler Wilms tumor	2
	Other tissues ^b	14
15		

^a Using the blast algorithm (Altschul et al., 1997, Nucleic Acids Res 25, 3389-402), a total of 98 human EST sequences were identified that matched the 3'UTR of the PAPP-A2 mRNA sequence. The distribution among tissues is based on the annotations of individual database entries (not listed).

^b EST sequences originated from pools of tissue, or from tissue represented by only one EST sequence.

25 6.4. Expression in mammalian cells of recombinant PAPP-A2 and variants of PAPP-A2

The following plasmid constructs were made:

30 a) pPA2: The cDNA sequence of pre-pro-PAPP-A2 encoding amino acids 1-1791 in expression vector pcDNA3.1+.

b) pPA2-KO: As pPA2, but Glu-734 of the active site of PAPP-A2 substituted with a Gln residue (E734Q).

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c) pPA2-mH: The expression vector pcDNA3.1/*Myc*-His(-)A containing the cDNA sequence of pre-pro-PAPP-A2 encoding amino acids 1-1791, not followed by a stop codon, but rather a *c-myc* and a His tag.

5 d) pPA2-KO-mH: As pPA2-mH, but with the E734Q substitution of pPA2-KO.

The three overlapping PAPP-A2 cDNA fragments (hom-N, the midregion, and hom-C) were used for the construction of a single contiguous cDNA sequence encoding PAPP-A2. The overlapping fragments were all contained in the vector pCR-BluntII-TOPO (Invitrogen) and referred to as p2N, p2Mid, and p2C, as detailed above (example 6.1). Clones of p2N and p2C were selected that had the proper orientation of the cDNA insert.

Construction of pPA2: The *NotI*-*BamHI* fragment was excised from p2C and cloned into pBluescriptIIISK+ (Stratagene) to obtain p2CBlue. The *NotI*-*SpeI* fragment was excised from p2N, and the *SpeI*-*BclI* fragment was excised from p2Mid. Those two fragments were ligated into the *NotI*/*BclI* sites of p2CBlue in one reaction to obtain p2NMidCBlue, containing the entire PAPP-A2 cDNA. The *NotI*-*ApaI* fragment of pBluescriptIIISK+ was excised and ligated into the *NotI*/*ApaI* sites of the mammalian expression vector pcDNA3.1+ (Invitrogen) to obtain a modified version of this vector, pcDNA-NA. The full length cDNA was then excised from p2NMidCBlue with *NotI* and *XhoI* and cloned into pcDNA-NA to obtain pPA2. All restriction sites used are in the multi cloning sites of the vectors, except for *SpeI* and *BclI*, both located in each of the two overlapping regions of the coding PAPP-A2 sequence stretches of p2N, p2Mid, and p2C (nt. 2365 and nt. 4203, respectively, of Figure 3).

Construction of pPA2-KO: The construct pPA2-KO is a variant of the pPA2 expression construct in which residue Glu-734 of the active site of PAPP-A2 was substituted with a Gln residue. Thus, the mutant is E734Q. The pPA2-KO construct was made by site directed mutagenesis using the method of overlap extension PCR (Ho et al., 1989, Gene 77, 51-9) with pPA2 as the template. In brief, outer primers were 5'-CGCTCAGGGAAGGACAAGGG-3' (5' end primer, nt. 976-995 of SEQ ID NO:1) and 5'-CTAGAAGGCACAGTCGAGGC-3' (3' end primer, nt. 1040-1021, sequence of vector pcDNA3.1+). Overlapping internal primers were 5'-TGTCCTCACTTGATGGATCATGGTGTCTGGTGTGG-3' (nt. 2210-2178 of SEQ ID

NO:1, nt. 2200 not C, but G resulting in E734Q) and 5'-

CCATCAAGTGGGACATGTTCTGGGAC-3' (nt. 2196-2221 of SEQ ID NO:1, nt.

2200 not G, but C resulting in E734Q). The resulting mutated fragment was digested with *XbaI* and *XhoI* and swapped into pPA2 to generate pPA2-KO. All PCRs were carried out with *Pfu* DNA polymerase (Stratagene), and all constructs were verified by sequence analysis.

Construction of pPA2-mH: Two primers (5'-GAGGGCCTGTGGACCCAGGAG-3', nt. 4906-4926 of SEQ ID NO:1, and 5'-

GACGTAAAGCTTCTGATTTTCTTCTGCCTTGG-3', nt. 5373-5354 of SEQ ID NO:1, preceded by a *HindIII* site, AAGCTT, and nt. GACGTA to facilitate cleavage of the

PCR product) were used in a PCR with pPA2 as the template to generate a nucleotide fragment encoding the C-terminal 156 residues of PAPP-A2 with the stop codon replaced by a *HindIII* site for in-frame ligation to expression vector. In brief, the PCR

product was digested with *EcoRI* and *HindIII* and cloned into the *EcoRI/HindIII* sites

of the vector-pcDNA3.1/*Myc*-His(-)A to generate pPA2C-mH. The *NotI-XbaI* fragment (encoding the N-terminal portion of PAPP-A2), and the *XbaI-EcoRI* fragment

(encoding the remaining central portion of PAPP-A2) were excised from pPA2 and ligated in one reaction into the *NotI/EcoRI* sites of pPA2C-mH. The resulting construct, pPA2-mH, encoded PAPP-A2 followed by residues KLGP, the *myc* epitope

(EQKLISEEDL), residues NSAVD, and six H-residues (amino acids are given as one letter code). A stop codon follows immediately after the six histidine residues.

Construction of pPA2-KO-mH: A variant of pPA2-mH was constructed with residue

Glu-734 substituted into a Gln residue: The *NotI-KpnI* fragment of pPA2-KO was excised and swapped into the *NotI-KpnI* sites of pPA2-mH, to generate pPA2-KO-mH.

Expression in mammalian cells: All constructs (pPA2, pPA2-KO, pPA2-mH, and pPA2-KO-mH) as well as empty expression vectors (pcDNA3.1+ and

pcDNA3.1/*Myc*-His(-)A) were transiently transfected into mammalian cells for expression of recombinant PAPP-A2 protein. Briefly, human embryonic kidney 293T

cells (293tsA1609neo) (DuBridge et al., 1987, *Mol Cell Biol* 7, 379-87) were maintained in high glucose DMEM medium supplemented with 10% fetal bovine serum, 2

mM glutamine, nonessential amino acids, and gentamicin (Life Technologies). Cells were plated onto 6 cm tissue culture dishes, and were transfected 18 h later by cal-

cium phosphate coprecipitation (Pear et al., 1993, Proc Natl Acad Sci U S A 90, 8392-6) using 10 µg of plasmid DNA prepared by QIAprep Spin Kit (Qiagen). After a further 48 h the supernatants were harvested, and replaced by serum-free medium (293 SFM II, Life Technologies) for another 48 h. The serum-free medium was harvested and cleared by centrifugation.

Analysis by Western blotting of recombinant protein resulting from transfection with the constructs pPA2-mH and pPA2-KO-mH, demonstrated that PAPP-A2 is secreted as a protein of 220 kDa (See Figure 2). Reduction of disulfide bonds did not cause a visible change in band migration. Thus, in contrast to PAPP-A, PAPP-A2 is secreted as a monomer.

6.5. Purification by affinity chromatography of tagged PAPP-A2

A metal chelate affinity column (2 ml, Pharmacia) was charged with nickel ions and loaded with serum-free medium (50 ml) from cells transiently transfected with pPA2-KO-mH (see example 6.4). After washing in PBS containing 1M NaCl, bound protein was eluted with 10 mM EDTA in PBS in fractions of 0.5 ml. PAPP-A2 containing fractions were located by SDS-PAGE (Figure 4, lane 5). This protein was not seen from medium of cells transfected with empty vector (mock transfectants) and treated in a parallel manner.

6.6. N-terminal sequence analysis of PAPP-A2

C-terminally tagged PAPP-A2 purified from medium of cells transfected with construct pPA2-KO-mH (see examples 6.4 and 6.5) was reduced and run on a 10-20% SDS gel, and further blotted onto PVDF membrane (ProBlott, Applied Biosystems). Bands of 4 lanes were excised and subjected to N-terminal sequence analysis on an Applied Biosystems 477A sequencer equipped with an on-line HPLC (Sottrup-Jensen, 1995, Anal Biochem 225, 187-8). The N-terminal sequence observed at a level of approximately 20 pmol was: Ser-Pro-Pro-Glu-Glu-Ser-Asn (SPPEESN), resulting from cleavage before Ser-234 of the PAPP-A2 polypeptide after R(230)VKK.

This confirms the prediction, that PAPP-A2, like PAPP-A, is synthesized as a prepro protein. The absence of an arginine residue in the P1 position, indicates that the

proprotein processing enzyme responsible for this cleavage is not furin, but likely another proprotein convertase (Nakayama, 1997, Biochem J 327, 625-35). Cleavage of proPAPP-A2 might have been predicted after R(196)QRR, which archetypically marks furin cleavage (Nakayama, 1997, Biochem J 327, 625-35). We cannot
 5 exclude that cleavage occurred at this site, and that the observed N-terminus results from further processing.

6.7. Cleavage of insulin-like growth factor binding protein (IGFBP)-5

10 Ligand blotting (Conover et al., 1993, J Clin Invest 91, 1129-37) with radiolabeled IGF-II (Bachem) was used to assay for activity against IGFBP-1 (from HepG2 conditioned medium), rIGFBP-2 (GroPep), rIGFBP-3 (gift of D. Powell), rIGFBP-4 (Austral), rIGFBP-5 (gift of D. Address), and rIGFBP-6 (Austral). Of the six binding proteins, IGFBP-5 showed complete cleavage (Figure 5). IGFBP-3 was partially de-
 15 graded (Figure 5). This cleavage was independent of the presence of IGF. Experiments were carried out with media from cells transfected with pPA2 or empty vector. - - -

For further analysis, recombinant IGFBP-5 was produced in mammalian cells. In brief, human placental oligo-dT primed cDNA (Overgaard et al., 1999, Biol Reprod
 20 61, 1083-9) was used as a template to amplify cDNA encoding human IGFBP-5 (Accession number M65062). Specific primers containing an *Xho*I site (5'-TCCGCTCGAGATGGTGTGCTCACC GCGGT-3') and a *Hind*III site (5'-CGATAAGCTTCTCAACGTTGCTGCTGTCG-3') were used, and the resulting PCR product was digested and cloned into the *Xho*I/*Hind*III sites of pcDNA3.1/*Myc*-His(-)
 25)A (Invitrogen). The construct encoded the full-length proIGFBP-5, immediately followed by residues KLGP, the *myc* epitope (EQKLISEEDL), residues NSAVD, and six H-residues (amino acids are given as one letter code). The construct was verified by sequence analysis. Plasmid DNA for transfection was prepared by QIAprep Spin Kit (Qiagen). Cell culture and expression of recombinant IGFBP-5 was per-
 30 formed as described above in Example 6.4.

Cleavage analysis was performed by Western blotting (Figure 6). Briefly, recombinant IGFBP-5 as contained in 5 microL cell culture medium was incubated with culture supernatants (10 microL) from cells transfected with pPA2, pPA2-KO, or empty
 35 expression expression vectors (see example 6.4). Phosphate buffered saline was

added to a final volume of 50 microL. After incubation at 37 degrees Celsius for 12 hours, 15 microL of the reaction mixture was separated by reducing 16% SDS-PAGE, blotted onto a PVDF membrane, and the C-terminal cleavage product was detected with monoclonal anti-*c-myc* (clone 9E19, ATTC) using peroxidase-conjugated secondary antibodies (P260, DAKO), and enhanced chemiluminescence (ECL, Amersham).

6.8. Inhibition of the activity of PAPP-A2

Various agents were analyzed for their ability to inhibit the proteolytic activity of PAPP-A2 against IGFBP-5. The experimental conditions were essentially as described in Example 6.7, except the agents to be tested were added (Figure 6). Agents found to have no effect on the proteolytic activity of PAPP-A2 further included PMSF and aprotinin.

6.9. Identification of the cleavage site in IGFBP-5

For cleavage site determination, purified rIGFBP-5 (Fig. 6, lane 7) was digested with purified PAPP-A2 and analyzed by SDS-PAGE (Fig. 6, lane 8). Edman degradation of blotted material showed that both distinct, visible degradation products (fig. 6, lane 8) contained the N-terminal sequence K(144)FVGGA (IGFBP-5 is numbered with the N-terminal Leu of the mature protein as residue 1). The two bands both represent intact C-terminal cleavage fragments, because they also contain the C-terminal *c-myc* tag (Fig. 6, lane 9); they are likely to be differently glycosylated, in accordance with the heterogeneity of purified rIGFBP-5 (Figure 6, lane 7). Both bands contained a second sequence at lower level (45%), L(1)GXFVH, corresponding to the N-terminal sequence of IGFBP-5. The absence of Ser, expected in the third cycle, was taken as evidence for carbohydrate substitution of Ser-3. O-linked glycan on the N-terminal cleavage fragment is likely to cause it to smear around the two distinct, C-terminal fragments. Sequence analysis on the reaction mixture (> 100 pmol) without SDS-PAGE separation showed only the same two IGFBP-5 sequences in equimolar amounts. Thus, PAPP-A2 cleaves IGFBP-5 at one site, between Ser-143 and Lys-144.

6.10. Tissues where PAPP-A2 may cause proteolysis of IGFBP-5

Proteolytic activity against IGFBP-5 has been widely reported from several sources, e.g. pregnancy serum (Claussen et al., 1994, Endocrinology 134, 1964-6), seminal plasma (Lee et al., 1994, J Clin Endocrinol Metab 79, 1367-72), culture media from smooth muscle cells (Imai et al., 1997, J Clin Invest 100, 2596-605), granulosa cells (Resnick et al., 1998, Endocrinology 139, 1249-57), osteosarcoma cells (Conover and Kiefer, 1993, J Clin Endocrinol Metab 76, 1153-9), and also from osteoblasts (Thraill et al., 1995, Endocrinology 136, 3527-33), and fibroblasts (Busby et al., 2000, J Biol Chem). In general, the proteinase responsible for cleavage of IGFBP-5 has remained unidentified.

The recent identification of PAPP-A as the IGFBP-4 proteinase in fibroblasts and osteoblasts (Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53), ovarian follicular fluid (Conover et al., 1999, J Clin Endocrinol Metab 84, 4742-5), pregnancy serum (Overgaard et al., 2000, J Biol Chem), and vascular smooth muscle cells (Bayes-Genis, A., Schwartz, R. S., Ashai, K., Lewis, D. A., Overgaard, M. T., Christiansen, M., Oxvig, C., Holmes, D. R., Jr., and Conover, C. A. Arterioscler. Thromb. Vasc. Biol., in press) firmly establishes PAPP-A and IGFBP-4 as an important functional pair in several systems. No other substrate as has been found for PAPP-A, and no other proteinase has been shown to cleave IGFBP-4 physiologically. It is therefore likely that the pair of PAPP-A2 and IGFBP-5 plays an analogous role in a number of the tissues mentioned above and/or elsewhere. Interestingly, incubating IGFBP-5 with smooth muscle cells conditioned medium resulted in cleavage between Ser-143 and Lys-144 (Imai et al., 1997, J Clin Invest 100, 2596-605), the same cleavage site as found here with PAPP-A2. This immediately suggests PAPP-A2 as an obvious candidate IGFBP-5 proteinase for this tissue.

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PATENT CLAIMS:

1. A purified polynucleotide selected from the group consisting of
 - 5 i) a polynucleotide comprising nucleotides 1 to 5376 of SEQ ID NO:1, corresponding to the coding sequence of PAPP-A2, as deposited with DSMZ under accession number DSM 13783; and
 - 10 ii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and
 - iii) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment
 - 15 a) has a proteolytic activity specific for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); or a derivative thereof, or any other substrate; and/or
 - 20 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
 - 25 c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide; and
 - 30 iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), said polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2, or a fragment thereof, wherein said fragment
 - 35 a) has a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or

b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

5 c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide; and

10 v) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iii) and (iv),

and the complementary strand of such a polynucleotide.

15 2. A purified polynucleotide according to claim 1 and comprising the coding sequence as shown in SEQ ID NO:1.

3. A polynucleotide according to claim 1 and encoding a polypeptide the amino acid sequence as shown in SEQ ID NO:2.

20

4. A polynucleotide according to claim 1 and encoding a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:2, wherein said fragment

25 a) has a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or

30 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

c) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor having an affinity for said polypeptide

35

5. A polynucleotide according to claim 1, wherein the complementary strand of said polynucleotide hybridizes, under stringent conditions, with a polynucleotide according to any of claims 2 to 4.
- 5 6. A polynucleotide according to claim 1 and comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide according to any of claims 3 and 4.
- 10 7. A polynucleotide according to claim 1, said polynucleotide comprising the complementary strand of a polynucleotide according to any of claims 2 to 6.
- 15 8. A polynucleotide according to any of the preceding claims operably linked to a further polynucleotide comprising nucleic acid residues 5377 to 8527 of SEQ ID NO:1, corresponding to a 3' untranslated region, or a fragment thereof, or SEQ ID NO:1.
-
- 20 9. A recombinant DNA molecule in the form of an expression vector comprising an expression signal operably linked to a polynucleotide according to any of claims 1 to 7.
10. A host organism transfected or transformed with the polynucleotide according to any of claims 1 to 8, or the vector according to claim 9.
- 25 11. Host organism according to claim 10, wherein said organism is a mammalian organism.
12. An isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:2, or a fragment thereof, wherein said fragment
- 30 i) has a proteolytic activity specific at least for Insulin Like Growth Factor Binding Protein 5 (IGFBP-5); and/or
- 35 ii) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

- iii) competes with a polypeptide having the amino acid sequence as shown in SEQ ID NO:2 for binding to a cell surface receptor with an affinity for said polypeptide.

5

13. Polypeptide according to claim 12, wherein the fragment comprises or essentially consists of amino acid residues 234 to 1791 corresponding to the mature part of PAPP-A2, including any processing variant thereof.

10

14. Polypeptide according to claim 12, wherein the fragment comprises or essentially consists of amino acid residues 1 to 233 corresponding to the prepro part of PAPP-A2.

15

15. Polypeptide according to claim 12, wherein the fragment comprises or essentially consists of amino acid residues 23 to 233 corresponding to the pro part of PAPP-A2.

20

16. Polypeptide according to claim 12, wherein the fragment comprises or essentially consists of amino acid residues 1 to 22 corresponding to the signal peptide or leader sequence of PAPP-A2.

25

17. Polypeptide according to any of claims 14 to 16 operably linked to the mature part of PAPP-A2 corresponding to amino acid residues 234 to 1791 of SEQ ID NO:2.

30

18. Polypeptide according to any of claims 12 to 17, wherein said polypeptide is a recombinant polypeptide.

19. Polypeptide according to any of claims 12 to 18, wherein the polypeptide is free of human proteins, or other proteins natively associated with said polypeptide.

35

20. A composition comprising i) the polynucleotide according to any of claims 1 to 8, and/or ii) the vector according to claim 9, and/or iii) the host organism according to any of claims 10 and 11, and/or iv) the polypeptide according to any of claims 12 to 19, in combination with a physiologically acceptable carrier.

5 21. A pharmaceutical composition comprising i) the polynucleotide according to any of claims 1 to 8, and/or ii) the vector according to claim 9, and/or iii) the host organism according to any of claims 10 and 11, and/or iv) the polypeptide according to any of claims 12 to 19, in combination with a pharmaceutically acceptable carrier.

10 22. A method for producing an antibody with specificity for the polypeptide according to claim 12, said method comprising the steps of

- i) providing a host organism,
- ii) immunizing the host organism with the polypeptide according to claim 10, and
- 15 iii) obtaining said antibody.

20 23. An antibody having specific binding affinity for a polypeptide according to claim 12.

24. Antibody according to claim 23, wherein said antibody is selected from the group consisting of monoclonal antibodies and polyclonal antibodies.

25 25. Antibody according to claim 24, wherein said antibody is monoclonal.

26. A method for producing the polypeptide according to claim 18, said method comprising the steps of

- 30 i) providing a suitable host organism,
- ii) transfecting or transforming the host organism provided in step i) with the polynucleotide according to any of claims 1 to 8, or the vector according to claim 9,

iii) culturing the host organism obtained in step ii) under conditions suitable for expression of the polypeptide encoded by the polynucleotide or the vector; and optionally

5 iv) isolating from the host organism the polypeptide resulting from recombinant expression by the host organism.

27. The method of claim 26, wherein said host organism is a mammalian cell.

10 28. A method for inhibiting and/or reducing expression of PAPP-A2 in a cell by means of anti-sense technology, said method comprising the steps of

i) providing the polynucleotide according to claim 7,

15 ii) transfecting or transforming a cell capable of expressing PAPP-A2 with said polynucleotide provided in step i),

iii) culturing the cell obtained in step ii) under conditions suitable for hybridization of the polynucleotide provided in step i) to a complementary polynucleotide in said cell involved in the expression of PAPP-A2, and

20

iv) inhibiting and/or reducing the expression of PAPP-A2 in said cell.

25 29. Method of claim 28, wherein the antisense polynucleotide and the complementary polynucleotide are co-expressed from distinct polynucleotide molecules.

30. A method for detecting PAPP-A2, or measuring the level of PAPP-A2, in a biological sample obtained from an individual, said method comprising the steps of

30 i) obtaining a biological sample from said individual,

ii) detecting PAPP-A2 in said sample by detecting

a) a polypeptide according to claim 12; and/or

b) a polynucleotide in the form of mRNA originating from PAPP-A2 expression, and/or

c) PAPP-A2 specific protease activity, preferably by detecting cleavage of IGFBP-5, a derivative thereof, or any other suitable substrate for PAPP-A2.

31. Method of claim 30, said method comprising the further step of comparing the PAPP-A2 or the level of PAPP-A2 detected in step ii) with a predetermined value selected from the group consisting of

i) a predetermined amount and/or concentration of PAPP-A2; and/or

ii) a predetermined amount and/or concentration of PAPP-A2 mRNA; and/or

iii) a predetermined PAPP-A2 specific protease activity.

32. Method of claim 31, wherein said predetermined value is indicative of a normal physiological condition of said individual.

33. The method of claim 30, wherein said biological sample is selected from the group consisting of blood, urine, pleural fluid, oral washings, tissue biopsies, and follicular fluid.

34. The method of claim 30, wherein said level of PAPP-A2 is measured as PAPP-A2 specific protease activity.

35. The method of claim 30, wherein said level of PAPP-A2 is measured as amount of PAPP-A2 protein.

36. The method of claim 30, wherein said level of PAPP-A2 is measured as amount of PAPP-A2 messenger RNA.

37. The method of claim 35, wherein said amount of PAPP-A2 protein is measured by immunochemical analysis.
38. The method of claim 37, wherein said amount of PAPP-A2 protein is detected by
5 at least one monoclonal antibody.
39. The method of claim 30, wherein said PAPP-A2 protein is detected in a complex comprising at least one additional component, preferably a polypeptide.
40. The method of claim 30, wherein said PAPP-A2 is detected as a PAPP-A2 monomer.
10
41. The method of claim 30, wherein said PAPP-A2 is detected as a PAPP-A2 dimer.
15
42. A method of diagnosing a clinical condition in an individual, said method comprising the steps of
15
- i) performing the method of any of claims 30 to 41, and
 - ii) diagnosing the clinical condition.
20
43. Method of claim 42, wherein said clinical condition is a fetal abnormality.
44. The method of claim 43, wherein said fetal abnormality is selected from the
25 group consisting of Trisomy 21, Trisomy 18, Trisomy 13, and Open Spina Bifida.
45. The method according to claim 43, wherein said fetal abnormality is ectopic pregnancy, open spina bifida, neural tube defects, ventral wall defects, Edwards
30 Syndrome, Patau's Syndrome, Turner Syndrome, Monosomy X or Klinefelter's Syndrome.
46. The method of claim 43, wherein said clinical condition is an altered growth state selected from the group consisting of a growth promoting state and a growth inhibiting state.
35

47. The method of claim 46, wherein said clinical condition is selected from the group consisting of restenosis, atherosclerosis, wound healing, fibrosis, myocardial infarction, osteoporoses, rheumatoroid arthritis, multiple myeloma, or cancer.

48. A method for detecting expression of a polynucleotide according to claim 1 in a biological sample, said method comprising the steps of:

- i) providing a biological sample putatively containing a polynucleotide according to claim 1, and
- ii) contacting the biological sample with a polynucleotide comprising a strand that is i) complementary to the polynucleotide according to claim 1 and ii) capable of hybridizing thereto, and
- iii) allowing hybridization to occur, and
- iv) detecting the hybridization complex obtained in step iii),

wherein the presence of the hybridization complex is indicative of the expression in the biological sample of the polynucleotide according to claim 1, or a fragment thereof.

49. A method for identifying an agent inhibiting the protease activity of PAPP-A2, said method comprising the steps of

- i) incubating a) the polypeptide according to claim 12 and b) a predetermined substrate for said polypeptide, and c) a putative inhibitory agent, and
- ii) determining if proteolysis of said substrate is inhibited.

50. The method of claim 49, wherein said substrate comprises a polypeptide.

51. The method of claim 50, wherein said substrate comprises an internally quenched fluorescent peptide.

5 52. The method of claim 50, wherein said substrate comprises or essentially consists of IGFBP-5, or a fragment thereof.

53. An inhibitory agent obtainable according to any to the method of any of claims 49 to 52.

10 54. Use of the inhibitory agent according to claim 53 in the manufacture of a medicament for treating a clinical condition in an individual in need of such treatment.

15 55. A method for identifying an agent enhancing the protease activity of PAPP-A2, said method comprising the steps of

20 i) incubating a) the polypeptide according to claim 12 and b) a predetermined substrate for said polypeptide, and c) a putative enhancer agent, and

ii) determining if proteolysis of said substrate is enhanced.

56. The method of claim 53, wherein said substrate comprises a polypeptide.

25 57. The method of claim 54, wherein said substrate comprises an internally quenched fluorescent peptide.

58. The method of claim 54, wherein said substrate comprises or essentially consists of IGFBP-5, or a fragment thereof.

30 59. An enhancing agent obtainable according to any to the method of any of claims 54 to 57.

60. Use of the enhancing agent according to claim 59 in the manufacture of a medicament for treating a clinical condition in an individual in need of such treatment.

5 61. A method of treatment by therapy of an individual, said method comprising the step of administrating to said individual i) the pharmaceutical composition according to claim 21, and/or ii) the inhibitory agent according to claim 53, and/or the enhancing agent according to claim 59.

62. A method for purification of PAPP-A2 or complexes of PAPP-A2 with other proteins, said method comprising the steps of

i) providing a polyclonal or monoclonal antibody with specific binding affinity for a polypeptide according to claim 12,

15

ii) - - - purifying PAPP-A2 by means of affinity chromatography.

ABSTRACT

The present invention provides nucleotide and amino acid sequences that identify and encode a new protein with homology to pregnancy-associated plasma protein-A (PAPP-A). We denote this protein PAPP-A2. The cDNA encoding PAPP-A2 was derived from human placenta. The present invention also provides for antisense molecules to the nucleotide sequences which encode PAPP-A2, expression vectors for the production of purified PAPP-A2, antibodies capable of binding specifically to PAPP-A2, hybridization probes or oligonucleotides for the detection of PAPP-A2-encoding nucleotide sequences, genetically engineered host cells for the expression of PAPP-A2, use of the protein to produce antibodies capable of binding specifically to the protein, methods for screening for pathologies in pregnant and non-pregnant patients that are based on detection of PAPP-A2 antigen in human body fluids or PAPP-A2-encoding nucleic acid molecules, use of the protein to screen for agents that alter the protease activity of PAPP-A2, use of the protein as a therapeutic target for such agents, and use of the protein as a therapeutic agent in relevant pathological states. Methods for screening for altered focal proliferation states in pregnant and/or non-pregnant patients, which include detecting levels of PAPP-A2, are also described. The present invention also provides the identification of a natural substrate of PAPP-A2, insulin-like growth factor binding protein (IGFBP)-5.

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Figure 1 (page 1 of 3)

ATGATGTGCTTAAAGATCCTAAGAATAAGCCTGGCGATTGCTGGGTGGGCACTCTGT	60
M M C L K I L R I S L A I L A G W A L C	(20)
TCTGCCAACTCTGAGCTGGGCTGGACACGCAAGAAATCCTTGGTTGAGAGGGAACCTG	120
S A N S E L G W T R K K S L V E R E H L	(40)
AATCAGGTGCTGTTGGAAGGAGAACGTTGTTGGCTGGGGCCAAAGGTTGGAAGCCAGA	180
N Q V L L E G E R C W L G A K V R R P R	(60)
GCTTCTCCACAGCATCACCTCTTGGAGTCTACCCAGCAGGGCTGGGAACTACCTAAGG	240
A S P Q H H L F G V Y P S R A G N Y L R	(80)
CCCTACCCCTGGGGGAGCAAGAAATCCATCATACAGGACGAGCAAAACGACACTGAA	300
P Y P V G E Q E I H H T G R S K P D T E	(100)
GGAAATGCTGTGAGCCTTGTCCCCAGACCTGACTGAAAATCCAGCAGGACTGAGGGGT	360
G N A V S L V P P D L T E N P A G L R G	(120)
GCAGTTGAAGAGCCGGCTGCCCATGGGTAGGGGATAGTCTATTGGGCAATCTGAGCTG	420
A V E E P A A P W V G D S P I G Q S E L	(140)
CTGGGAGATGATGACGCTTATCTCGGCAATCAAAGATCCAAGAGTCTTAGGTGAGGCC	480
L G D D D A Y L G N Q R S K E S L G E A	(160)
GGGATTGAGAAAGGCTCAGCCATGGCTGCCACTACTACCACCGCCATTTTCAACCCCTG	540
G I Q K G S A M A A T T T A I F T T L	(180)
AACGAACCCAAACAGAGACCCAAAGGAGGGGCTGGGCCAAGTCCAGGACGCTCGCAA	600
N E P K P E A T Q R R G W A K S R Q R R Q	(200)
GTGTGAAGAGGCGGGCGGAAGATGGGCGAGGAGTCCGGTATCTCTTACATTTCAA	660
V W K R R A E D G Q G D S G I S S H F Q	(220)
CCTTGGCCCAAGCATTCCCTTAAACACAGGGTCAAAAAGAGTCCACCGAGGAAAGCAAC	720
P W P K H S L K H R V K K S P P E E S N	(240)
CAAAATGGTGGAGGGGCTCCTACCGAGAAGCAGAGACCTTAACTCCCAAGTAGGACTG	780
Q N G G A G E G S Y R E A E T F N S Q V G L	(260)
CCCATCTTACTTCTCTGGGAGGCGGAGCGGCTGCTGCTGCTCCAGAAGTGCTGGCT	840
P I L Y F S G R R E R L L L R P E V L A	(280)
GAGATCCCCGGGAGGCGTTACAGTGGGAAGCCTGGGTTAAACCGGAGGAGGACAGAAC	900
E I P R E A F T V E A W V K P E G G Q N	(300)
AACCCAGCCATCATCGCAGGTGTGTTTGATAACTGCTCCACACTGTGAGTACAAAGGC	960
N P A I I A G V F D N C S H T V S D K G	(320)
TGGGCCCTGGGGATCCGCTCAGGGAAGGACAAGGGAAGCGGGATGCTCGCTTCTTCTTC	1020
W A L G I R S G K D K G K R D A R F F F	(340)
TCCCTCTGCACCGCGCTGAAGAAAGCCACCATCTTGATTAGCCACAGTCGCTACCAA	1080
S L C T D R V K K A T I L I S H S R Y Q	(360)
CCAGGCACATGGACCATGTGGCAGCCACTTACGATGGACGGCACATGGCCCTGTATGTG	1140
P G T W T H V A A T Y D G R H M A L Y V	(380)
GATGGCACTCAGGTGGCTAGCAGTCTAGACAGTCTGGTCCCCTGAACAGCCCTTCATG	1200
D G T Q V A S S L D Q S G P L N S P F M	(400)
GCATCTTGCCGCTCTTGTCTCTGGGGGAGACAGCTCTGAGGATGGGCACTATTTCCGT	1260
A S C R S L L L G G D S S E D G H Y F R	(420)
GGACACCTGGGCACACTGGTTTTCTGTCGACCGCCCTGCCACAAAGCCATTTTCAGCAC	1320
G H L G T L V F W S T A L P Q S H F Q H	(440)
AGTTCTCAGCATTCAAGTGGGAGGAGGAAGCGACTGACTTGGTCTGACAGCGAGCTTT	1380
S S Q H S S G E E E A T D L V L T A S F	(460)
GAGCCTGTGAACACAGAGTGGGTTCCCTTTAGAGATGAGAAGTACCCACGACTTGAGGTT	1440
E P V N T E W V P F R D E K Y P R L E V	(480)
CTCCAGGGCTTTGAGCCAGAGCCTGAGATTCTGTCGCTTTGCAGCCCCCACTCTGTGGG	1500
L Q G F E P E P E I L S P L Q P P L C G	(500)
CAAAAGTCTGTGACAAATGGAATTGATCTCCAGTACAAATGGATACTGGCCCTTCGG	1560
Q T V C D N V E L I S Q Y N G Y W P L R	(520)
GGAGAGAAGGTGATACGCTACAGGTGGTGAACATCTGTGATGATGAGGGCCTAAACCCC	1620
G E K V I R Y Q V V N I C D D E G L N P	(540)
ATTGTGAGTGAGGAGCAGATTCTGCTGCAGCAGGAGGCACTGAATGAGGCGCTTCAGCCG	1680
I V S E E Q I R L Q H E A L N E A F S R	(560)
TACAACATCAGCTGGCAGCTGAGCGTCCACAGGTCCACAATTCCACCTGCGACACCGG	1740
Y N I S W Q L S V H Q V H N S T L R H R	(580)
GTTGTGCTTGTGAACGTGAGCCGCAAGATTGGCAATGACCATTGTGACCCCGAGTGT	1800
V V L V N C E P S K I G N D H C D P E C	(600)

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Figure 1 (page 2 of 3)

GAGCACCCACTCACAGGCTATGATGGGGGTGACTGCCGCCTGCAGGGCCGCTGCTACTCC	1860
E H P L T G Y D G G D C R L Q G R C Y S	(620)
TGGAACCGCAGGGATGGGCTCTGTACGTGGAGTGTAAACAACATGCTGAACGACTTTGAC	1920
W N R R D G L C H V E C N N M L N D F D	(640)
GACGGAGACTGCTGCGACCCCGAGGTGGCTGATGTGCGCAAGACCTGCTTTGACCCTGAC	1980
D G D C C D P Q V A D V R K T C F D P D	(660)
TCACCCAAGAGGGCATACTAGTGTGAAGGAGCTGAAGGAGGCCCTGCAGCTGAACAGT	2040
S P K R A Y M S V K E L K E A L Q L N S	(680)
ACTCACTTCTCAACATCTACTTTGCCAGCTCAGTGCGGGAAGACCTTGACAGGTGCTGCC	2100
T H F L N I Y F A S S V R E D L A G A A	(700)
ACCTGGCCTTGGGACAAGGACGCTGTCACTCACCTGGGTGGCATTGTCTCAGCCCAGCA	2160
T W P W D K D A V T H L G G I V L S P A	(720)
TATTATGGGATGCTGGCCACCCGACACCATGATCCATGAAGTGGGACATGTTCTGGGA	2220
Y Y G M P G C H T D T M I H E V G H V L G	(740)
CTCTACCATGTCTTTAAAGGAGTCAGTGAAAGAGAATCCTGCAATGACCCCTGCAAGGAG	2280
L Y H V F K G V S E R E S C N D P C K E	(760)
ACAGTGCCATCCATGGAAACGGGAGACCTCTGTGCCGACACCCGCCCACTCCCAAGAT	2340
T V P S M E T G D L C A D T A P T P K S	(780)
GAGCTGTGCCGGGAACAGAGCCCACTAGTGACACCTGTGGCTTCACTCGCTTCCCAGGG	2400
E L C R E P E P T S D T C G F T R F P G	(800)
GCTCCGTTACCAACTACATGAGCTACACGGATGATAACTGCAGTACAACTTCACTCCT	2460
A P F T N Y M S Y T D D N C T D N F T P	(820)
AACCAAGTGGCCCAATGCATTGCTATTTGGACCTAGTCTATCAGCAGTGGACTGAAAGC	2520
N Q V A R M H C Y L D L V Y Q Q W T E S	(840)
AGAAAGCCCAACCCCATCCCCATTCCACCTATGGTCATCGGACAGACCAACAAGTCCCTC	2580
R K P T P I P I P P M V I G Q T N K S L	(860)
ACTATCCACTGGCTGCCTCCTATTAGTGGAGTTGTATATGACAGGGCCTCAGGCAGCTTG	2640
T I H W L P P I S G V V Y D R A S G S L	(880)
TGTGGCGCTTGCAGATGAAGATGGGACCTTTCTGTAGTATGTGCACACAGCTTCTCCCGG	2700
C G A C T E D G T F R Q Y V H T A S S R	(900)
CGGGTGTGTGACTCCTCAGGTTATTGGACCCAGAGGAGGCTGTGGGGCCTCCTGATGTG	2760
R V C D S S G Y W T P E E A V G P P D V	(920)
GATCAGCCCTGCGAGCCAAGCTTACAGGCCTGGAGCCCTGAGGTCCACCTGTACCACATG	2820
D Q P C E P S L Q A W S P E V H L Y H M	(940)
AACATGACGGTCCCCTGCCCAAGAGGCTGTAGCTTGGAGCTGCTCTTCCAACACCCG	2880
N M T V P C P T E G C S L E L L F Q H P	(960)
GTCCAAGCCGACACCTCACCTGTGGGTCACTTCTTCTTATGAGTCTCGCAGGTC	2940
V Q A D T L T L W V T S F F M E S S Q V	(980)
CTCTTTGACACAGAGATCTTGCTGGAACAAGGAGTCAGTGCACCTGGGCCCTTAGAC	3000
L F D T E I L L E N K E S V H L G P L D	(1000)
ACTTTCTGTGACATCCCACTCACCATCAAACCTGCACGTGGATGGGAAGGTGTGGGGGTG	3060
T P C D I P L T I K L H V D G K V S G V	(1020)
AAAGTCTACACCTTTGATGAGAGGATAGAGATTGATGCAGCACTCCTGACTTCTCAGCCC	3120
K V Y T F D E R I E I D A A L L T S Q P	(1040)
CACAGTCCCTGTGTCTGGCTGCAGGCCTGTGAGGTACCAGGTTCTCCGATCCCCA	3180
H S P L C S G C R P V R Y Q V L R D P P	(1060)
TTTGCCAGTGGTTTGGCCGTGGTGACACATTCTCACAGGAAGTTACGGACGTGGAG	3240
F A S G L P V V V T H S H R K F T D V E	(1080)
GTCACACCTGGACAGATGTATCAGTACCAAGTTCTAGCTGAAGCTGGAGGAGAACTGGGA	3300
V T P G Q M Y Q Y Q V L A E A G G E L G	(1100)
GAAGCTTCGCTCCTCTGAACCACATTCAATGGAGCTCCTTATTGTGGAGATGGGAAGGTG	3360
E A S P P L N H I H G A P Y C G D G K V	(1120)
TCAGAGAGACTGGGAGAAGAGTGTGATGATGGAGACCTTGTGAGCGGAGATGGCTGCTCC	3420
S E R L G E E C D D G D L V S G D G C S	(1140)
AAGGTGTGTGAGCTGGAGGAAGTTTCAACTGTGTAGGAGAGCCAAGCCTTTGCTACATG	3480
K V C E L E E G F N C V G E P S L C Y M	(1160)
TATGAGGGAGATGGCATATGTGAACCTTTTGAAGAAAAACAGCATTGTAGACTGTGGC	3540
Y E G D G I C E P F E R K T S I V D C G	(1180)
ATCTACACTCCCAAGGATACTTGGATCAATGGGCTACCGGGCTTACTCCTCTCATGAA	3600
I Y T P K G Y L D Q W A T R A Y S S H E	(1200)

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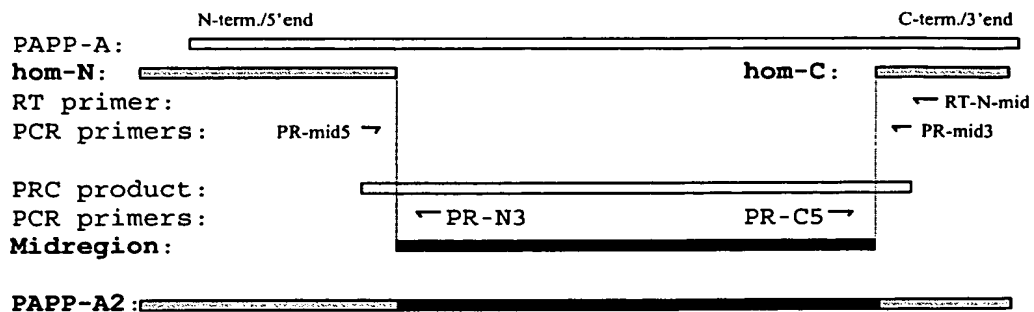
Figure 1 (page 3 of 3)

GACAAGAAGAAGTGTCTGTTTCCTTGGTAACCTGGAGAACCTCATTCCCTAATTTGCACA	3660
D K K K C P V S L V T G E P H S L I C T	(1220)
TCATACCATCCAGATTTACCCAACCACCGTCCCCCTAACTGGCTGGTTTCCCTGTGTGCC	3720
S Y H P D L P N H R P L T G W F P C V A	(1240)
AGTGAAAATGAAACTCAGGATGACAGGAGTGAACAGCCAGAAGGTAGCCTGAAGAAAGAG	3780
S E N E T Q D D R S E Q P E G S L K K E	(1260)
GATGAGGTTTGGCTCAAAGTGTGTTTCAATAGACCAGGAGAGGCCAGAGCAATTTTATT	3840
D E V W L K V C F N R P G E A R A I F I	(1280)
TTTTTGACAACTGATGGCTAGTTCCCGGAGAGCATCAGCAGCCGACAGTACTCTCTAC	3900
F L T T D G L V P G E H Q Q P T V T L Y	(1300)
CTGACCGATGTCCGTGGAAGCAACCACTCTCTTGGAACTATGGACTGTGATGCCAGCAT	3960
L T D V R G S N H S L G T Y G L S C Q H	(1320)
AATCCACTGATTATCAATGTGACCCATCACCAGAATGTCTTTTCCACCATACCACCTCA	4020
N P L I I N V T H H Q N V L F H H T T S	(1340)
GTGCTGTGAATTTCTCATCCCCACGGGTCGGCATCTCAGCTGTGGCTCTAAGGACATCC	4080
V L L N F S S P R V G I S A V A L R T S	(1360)
TCCCGCATTTGGTCTTTCGGCTCCCACTGACATCTCAGAGGACGAGGGGAGCAATCAT	4140
S R I G L S A P S N C I S E D E G Q N H	(1380)
CAGGGACAGAGCTGTATCCATCGGCCTGTGGGAAGCAGGACAGCTGCCGTCTATGCTG	4200
Q G Q S C I H R P C G K Q D S C P S L L	(1400)
CTTGATCATGCTGATGTGGTGAACCTGTACCTCTATAGGCCAGGTCTCATGAAGTGTGCT	4260
L D H A D V N C T S I G P G L M K C A	(1420)
ATCACTTGTCAAAGGGGATTGCCCCTCAGGCCAGCAGTGGGCAGTACATCAGGCCATG	4320
I T C Q R G F A L Q A S S G Q Y I R P M	(1440)
CAGAAGGAAATCTGCTCACATGTTCTTCTGGGCACTGGGACCAGAATGTGAGCTGCCTT	4380
Q K E I L L T C S S G H W D Q N V S C L	(1460)
CCCGTGGACTGCGGTGTTCGACCCGCTCTTGGTGAACATGCAAACTTCTCTGCTCA	4440
P V D C G V P D P S L V N Y A N F S C S	(1480)
GAGGGAACAAATTTCTGAAACGCTGCTCAATCTCTTGTGTCCACAGCCAAGCTGCAA	4500
E G T K F L K R C S I S C V P P A K L Q	(1500)
GGACTGAGCCCATGGCTGACATGTCTTGAAGATGGTCTCTGGTCTCTCCCTGAAGTCTAC	4560
G L S D T C L D G L W S L P E V Y	(1520)
TGCAAGTTGGAGTGTGATGCTCCCCCTATTATTCTGAATGCCAACTGCTCTGCCTCAC	4620
C K L E C D A P P I I L N A N L L L P H	(1540)
TGCCTCCAGGACAACACGACGTGGGCACCATCTGCAAAATATGAATGCAAACAGGGTAC	4680
C L Q D N H D V G T I C K Y E C K P G Y	(1560)
TATGTGGCAGAAAGTGACAGGGTAAAGTCAAGGAACAAGCTCCTGAAGATACAATGCCTG	4740
Y V A E S A E G K V R N K L L K I Q C L	(1580)
GAAGGTGGAATCTGGGAGCAAGGCAGCTGCATTCTGTGGTGTGTGAGCCACCCCTCCT	4800
E G G I W E Q G S C I P V V C E P P P P	(1600)
GTGTTGAAGGCATGTATGAATGTACCAATGGCTTCAGCCTGGACAGCCAGTGTGTGCTC	4860
V F E G M Y E C T N G F S L D S Q C V L	(1620)
AACTGTAAACAGGAACGTGAAAAGCTTCCCATCCTCTGCACTAAAGAGGGCCTGTGGACC	4920
N C N Q E R E K L P I L C T K E G L W T	(1640)
CAGGAGTTTAAGTTGTGTGAGAATCTGCAAGGAGAATGCCACCACCCCTCAGAGCTG	4980
Q E F K L C E N L Q G E C P P P P S E L	(1660)
AATTCTGTGGAGTACAAATGTGAACAAGGATATGGGATTGGTGCACTGTGTTCCCCATTG	5040
N S V E Y K C E Q G Y G I G A V C S P L	(1680)
TGTGTAATCCCCCAGTGACCCCGTGATGCTACCTGAGAATATCACTGTGACACTCTG	5100
C V I P P S D P V M L P E N I T A D T L	(1700)
GAGCACTGGATGGAACCTGTCAAAGTCCAGAGCATTGTGTGCACTGGCCGGCTCAATGG	5160
E H W M E P V K V Q S I V C T G R R Q W	(1720)
CACCCAGACCCCGTCTTAGTCCACTGCATCCAGTCATGTGAGCCCTTCCAAGCAGATGGT	5220
H P D P V L V H C I Q S C E P F Q A D G	(1740)
TGGTGTGACACTATCAACAACCGAGCCTACTGCCACTATGACGGGGGAGACTGCTGCTCT	5280
W C D T I N N R A Y C H Y D G G D C C S	(1760)
TCCACACTCTCTCCAAGAAGGTCAATTCATTGCTGCTGACTGTGACCTGGATGAGTGC	5340
S T L S S K K V I S P A A D C D L D E C	(1780)
ACCTGCCCCGACCCCAAGGCAGAAGAAATCAGTAA	5376
T C R D P K A E E N Q *	(1791)

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Figure 2 (page 1 of 1)



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Figure 3 (page 1 of 1)

PA2 mmElkilrisailagwalEsaNSELGWTRKKSLVEREHLNOVLLEGERCWLGAKVRRPRASPOHHLFGVYPSRAGNYLRPYVGEQEIHHTGRSKPDTEGNAVSLVPPDLTENPAGLRG 120
PA mrlwsvvhlglislaalgEglaERPRRARRDRPRAGRPPRAAGPATCATRGPPRLAAAAAAGRAWEAVRVPRRQOR----- 80

N-terminal residue of mature PAPP-A2 (Ser-234)↓

PA2 AVEEPAAPVWGDSPIGQSELLGDDDAYLGNQRSKESLGEAGIQKGSAMAATTTTAIFTTLNEPKPETQRRGWAKSRQRRQVWKRRAEDGQDGSIGSSHFPQWPWKHSLKHRVKKSPPEESN 240
PA -----

PA2 QNGGEGSYREAETFNQSOVGLPILYFSGRRERLLLREPEVLAEIPREAFTEAMVKPEGGONNPAIAGVFDNCSHTVSDKGWALGIRSGDKGKRDAFFFSLTDRVKKATILISHSYQ 360
PA -----EARGATEEPSPPSRALYFSGRGEOLRVLRADL-ELPRDAFTLQVWLRAGGORSFAVITGLYDKSYISDRGWVVGIIHTISDONKDPYFFSLKTDRAQVTTINAHRSYL 192

↑ N-terminal residue of mature PAPP-A (Glu-81)

PA2 PGTWTHVAATYDGRHMAHYVDGTOVASSLDSGGLNSPFMASEFSLLLGGDSSEGHYFRHGLTLVFNSTALPOSHFOHSHQSSGEEATDLVLTAQFEPVNTWVPPRDEKYPRLEV 480
PA PGOWVYLAATYDGGFMKLYVNGAQAQVATSGEQQVGIFSLPTQKQKVLMLGG--SALNNHYRGYIEHFSLWKVARTQREILSDMETHGAHTALPOLLQENWDNVKHAMSPMKDSSPKVEF 310

PA2 LOGFEPEPEILSPLOPPIEGOTVEDNVELISQYNGYVPLRGEKVIRYQVNNICDDEGLNPVSEEQIRLQHEALNEAFSRYNISQWLSVHQVHNSTLRHRVVLVNEPSKIGNDHCDPEG 600
PA SNAHG--FLLDTSLEPPIEGOTLEDNTEVIASYNQLSSFRQPKVVRVYVNVLYEDDHKNPTVTREQVDFQHQHQLAEAFQYINISWELDVLEVSNSSLRRRLILANCDISKIGDENCDPEG 428

LNR1

PA2 EHLPLTGYDGGDGR-LOGHGYSNRRRDGLCHVECNMMLNDFDDGDCDPOVADVRKTCFDPDPSPKRAYMSVKELKEALQNSTHFLNIYFASSVREDLAGAATWPDKDAVTHLGGIVLSP 719
PA NHTLTGHDDGGDGRHLRHRFAVKKHONGVCDMDNYERFNDGGECDPEITNVTOTCFDPDPSPHRAYLDVNLKNIKLDGSHLNIFFAKSSEELAGVATWPDKEALMHLGGIVLNP 548

LNR2

PA2 AYYGMPGHTDTMIHEVGHVGLGLYHVFQVYSERESCDNDFCKETVPSMETGDLADTAPTKESELCREPEPTSDTCGTFRFGAPFTNYMSYTDNCTDNFTPNQVARMHGYLDLVYOONTE 839
PA SFYGMGHTHTMIHEIGHSLGLYHVFQVYSEIQCSDNDFCKETVPSMETGDLADTNPAPKHKSCGDPGPGNDTCGFHSFNTPNYMSYADDCTDSFTPNQVARMHGYLDLVYOGNOP 668

PA2 SRKPTPIPIPPMVIQOTNKSLLTIHWLPPISGVVYDRAGSILCGATIEDGTFRQYVHTASSRVCDSGGYWTPEEAVGPPVDQPCFESLQAWSPEVHYHMNMVTPCP-TEGSLLELLFO 958
PA SRKPAPVALAPOVLGHTTDSVTLEWFFPIDGHFFERELGSACHILEGRILVQYASNASSPMPCSPSGHWSPREAEGHPDVEQPCKSSVRTWSPNSAVNPHTVPAPPEPOGYLELEFL 788

PA2 HPVQADTLTLWMT--SFFMESSQVLFDTEILLENKESVHLGPLDTFCIDPLTIKLH-VDGKVSQVGVYTFDERIEIDAALLTSQPHSPLESCGRFVRYQVLRDPPFASGLPVVTHSHRK 1075
PA YPLVPESLTIWVTFVSTDDWSGAVNDIKLLAVSGKNISLGPNVFCIDVPLTIRLWDVGEEVYGIQIYTLDEHLEIDAAMLTSTADTPLDCKPLKYKVRDPPLOMDVASIL-HLNRK 907

PA2 FTDVEVTPGQMYQYVLAEEAGGELGEASPLNHHGAPYCGDGKVSERLGEEDDGLVSGDGSQVFELEEGFNVGEPSTGYMYEGDGIPEFFERKTSIVDEGIYTPKGYLDQWATRA 1195
PA FVDMDLNLGSVYQYVWITISGTESEPSPAVYIHRGYCGDGIQKDGEGDDMNKINGDGSLEFCROEVSFNGIDEPSRQYFHDGQGVDEFEQKTSIKDGVYTPQGFLDQWASNA 1027

PA2 YSSHEDKKKCPVSLVTGEPHS-LIETSYHPDLNHRPLTGWFHVASENETQDRSEQPEGSKKKEDEVMLKVDFNRPGEARAFIFLTTDGLVPGEHQOQPTVTLTLTDVRGSNNHSLGT 1314
PA SVSHQDOOCPGWVVIIGAPAAQVCRKTVIDLSEGISQAHAWYCTISYPYSLAOTT-----FWLRAYFSQPMVAAAVIVHLVTDGTYYGQKQETISVOLLDTKQDSHDLGLH 1135

PA2 GLSCQHNPLIINVTHHONVLFHHTSVLLNLFSSPRVGISAVALRTSSRIGLSAPSNCISEDEQNHQGSIIHRRFGKQDSFSLLLDHADVNTSISGPLMKCAITGQRFALQASSG 1434
PA VLSQRNNPLIIPVHDLQOPFYHSAQVRFSSPLVAISGVALRSFDFPVTLSQO-RGETYSPAEOQSVHFAEKTDCPELAVENASLNSSSDRYHGAQTVSRTGYVLQIRRD 1253

SCR1

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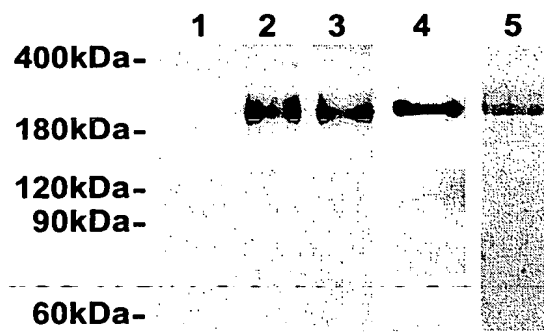
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Figure 4 (page 1 of 1)



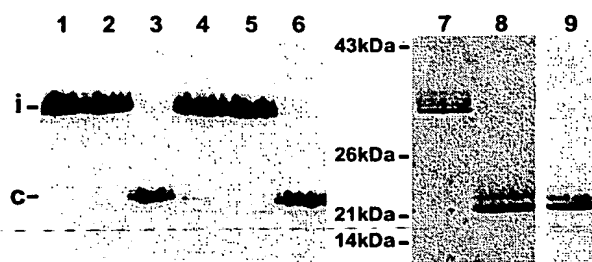
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Varemærkestyrelsen

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Figure 6 (page 1 of 1)



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Figure 7 (page 1 of 2)

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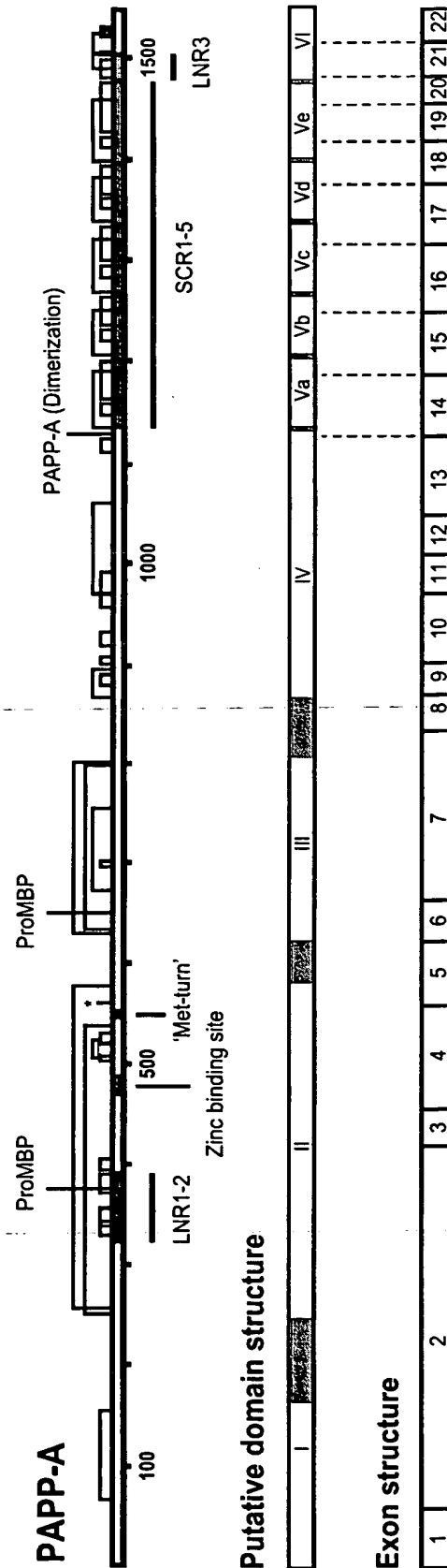
Figure 7 (page 2 of 2)

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Figure 8 (page 1 of 1)



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gtg gct agc agt cta gac cag tct ggt ccc ctg aac agc ccc ttc atg	Val Ala Ser Ser Leu Asp Gln Ser Gly Pro Leu Asn Ser Pro Phe Met	1200		
	155 160 165			
gca tct tgc cgc tct ttg ctc ctg ggg gga gac agc tct gag gat ggg	Ala Ser Cys Arg Ser Leu Leu Leu Gly Gly Asp Ser Ser Glu Asp Gly	1248		
	170 175 180			
cac tat ttc cgt gga cac ctg ggc aca ctg gtt ttc tgg tcg acc gcc	His Tyr Phe Arg Gly His Leu Gly Thr Leu Val Phe Trp Ser Thr Ala	1296		
	185 190 195			
ctg cca caa agc cat ttt cag cac agt tct cag cat tca agt ggg gag	Leu Pro Gln Ser His Phe Gln His Ser Ser Gln His Ser Ser Gly Glu	1344		
	200 205 210 215			
gag gaa gcg act gac ttg gtc ctg aca gcg agc ttt gag cct gtg aac	Glu Glu Ala Thr Asp Leu Val Leu Thr Ala Ser Phe Glu Pro Val Asn	1392		
	220 225 230			
aca gag tgg gtt ccc ttt aga gat gag aag tac cca cga ctt gag gtt	Thr Glu Trp Val Pro Phe Arg Asp Glu Lys Tyr Pro Arg Leu Glu Val	1440		
	235 240 245			
ctc cag ggc ttt gag cca gag cct gag att ctg tcg cct ttg cag ccc	Leu Gln Gly Phe Glu Pro Glu Pro Glu Ile Leu Ser Pro Leu Gln Pro	1488		
	250 255 260			
cca ctc tgt ggg caa aca gtc tgt gac aat gtg gaa ttg atc tcc cag	Pro Leu Cys Gly Gln Thr Val Cys Asp Asn Val Glu Leu Ile Ser Gln	1536		
	265 270 275			
tac aat gga tac tgg ccc ctt cgg gga gag aag gtg ata cgc tac cag		1584		

Tyr 280	Asn	Gly	Tyr	Trp	Pro 285	Leu	Arg	Gly	Glu	Lys 290	Val	Ile	Arg	Tyr	Gln 295	
gtg	gtg	aac	atc	tgt	gat	gat	gag	ggc	cta	aac	ccc	att	gtg	agt	gag	1632
Val	Val	Asn	Ile	Cys 300	Asp	Asp	Glu	Gly	Leu 305	Asn	Pro	Ile	Val	Ser	Glu 310	
gag	cag	att	cgt	ctg	cag	cac	gag	gca	ctg	aat	gag	gcc	ttc	agc	cgc	1680
Glu	Gln	Ile	Arg 315	Leu	Gln	His	Glu	Ala 320	Leu	Asn	Glu	Ala	Phe 325	Ser	Arg	
tac	aac	atc	agc	tgg	cag	ctg	agc	gtc	cac	cag	gtc	cac	aat	tcc	acc	1728
Tyr	Asn	Ile 330	Ser	Trp	Gln	Leu	Ser 335	Val	His	Gln	Val	His	Asn	Ser	Thr 340	
ctg	cga	cac	cgg	gtt	gtg	ctt	gtg	aac	tgt	gag	ccc	agc	aag	att	ggc	1776
Leu	Arg	His 345	Arg	Val	Val	Leu 350	Val	Asn	Cys	Glu	Pro 355	Ser	Lys	Ile	Gly	
aat	gac	cat	tgt	gac	ccc	gag	tgt	gag	cac	cca	ctc	aca	ggc	tat	gat	1824
Asn	Asp	His	Cys	Asp	Pro 365	Glu	Cys	Glu	His	Pro 370	Leu	Thr	Gly	Tyr	Asp 375	
ggg	ggt	gac	tgc	cgc	ctg	cag	ggc	cgc	tgc	tac	tcc	tgg	aac	cgc	agg	1872
Gly	Gly	Asp	Cys 380	Arg	Leu	Gln	Gly	Arg	Cys 385	Tyr	Ser	Trp	Asn	Arg	Arg 390	
gat	ggg	ctc	tgt	cac	gtg	gag	tgt	aac	aac	atg	ctg	aac	gac	ttt	gac	1920
Asp	Gly	Leu 395	Cys	His	Val	Glu	Cys	Asn 400	Asn	Met	Leu	Asn	Asp 405	Phe	Asp	
gac	gga	gac	tgc	tgc	gac	ccc	cag	gtg	gct	gat	gtg	cgc	aag	acc	tgc	1968
Asp	Gly	Asp 410	Cys	Cys	Asp	Pro	Gln	Val	Ala	Asp	Val	Arg	Lys	Thr	Cys	
ttt	gac	cct	gac	tca	ccc	aag	agg	gca	tac	atg	agt	gtg	aag	gag	ctg	2016
Phe	Asp	Pro 425	Asp	Ser	Pro	Lys 430	Arg	Ala	Tyr	Met	Ser 435	Val	Lys	Glu	Leu	
aag	gag	gcc	ctg	cag	ctg	aac	agt	act	cac	ttc	ctc	aac	atc	tac	ttt	2064
Lys	Glu	Ala 440	Leu	Gln	Leu 445	Asn	Ser	Thr	His	Phe 450	Leu	Asn	Ile	Tyr	Phe 455	
gcc	agc	tca	gtg	cgg	gaa	gac	ctt	gca	ggt	gct	gcc	acc	tgg	cct	tgg	2112
Ala	Ser	Ser 460	Val	Arg	Glu	Asp	Leu	Ala	Gly 465	Ala	Ala	Thr	Trp	Pro	Trp 470	
gac	aag	gac	gct	gtc	act	cac	ctg	ggt	ggc	att	gtc	ctc	agc	cca	gca	2160
Asp	Lys	Asp 475	Ala	Val	Thr	His	Leu	Gly 480	Gly	Ile	Val	Leu	Ser 485	Pro	Ala	
tat	tat	ggg	atg	cct	ggc	cac	acc	gac	acc	atg	atc	cat	gaa	gtg	gga	2208
Tyr	Tyr	Gly 490	Met	Pro	Gly	His	Thr 495	Asp	Thr	Met	Ile	His	Glu	Val	Gly 500	
cat	gtt	ctg	gga	ctc	tac	cat	gtc	ttt	aaa	gga	gtc	agt	gaa	aga	gaa	2256
His	Val	Leu 505	Gly	Leu	Tyr	His 510	Val	Phe	Lys	Gly	Val 515	Ser	Glu	Arg	Glu	

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Ser Cys Asn Asp Pro Cys Lys Glu Thr Val Pro Ser Met Glu Thr Gly	
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gac ctc tgt gcc gac acc gcc ccc act ccc aag agt gag ctg tgc cgg	2352
Asp Leu Cys Ala Asp Thr Ala Pro Thr Pro Lys Ser Glu Leu Cys Arg	
540 545 550	
gaa cca gag ccc act agt gac acc tgt ggc ttc act cgc ttc cca ggg	2400
Glu Pro Glu Pro Thr Ser Asp Thr Cys Gly Phe Thr Arg Phe Pro Gly	
555 560 565	
gct ccg ttc acc aac tac atg agc tac acg gat gat aac tgc act gac	2448
Ala Pro Phe Thr Asn Tyr Met Ser Tyr Thr Asp Asp Asn Cys Thr Asp	
570 575 580	
aac ttc act cct aac caa gtg gcc cga atg cat tgc tat ttg gac cta	2496
Asn Phe Thr Pro Asn Gln Val Ala Arg Met His Cys Tyr Leu Asp Leu	
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gtc tat cag cag tgg act gaa agc aga aag ccc acc ccc atc ccc att	2544
Val Tyr Gln Gln Trp Thr Glu Ser Arg Lys Pro Thr Pro Ile Pro Ile	
600 605 610 615	
cca cct atg gtc atc gga cag acc aac aag tcc ctc act atc cac tgg	2592
Pro Pro Met Val Ile Gly Gln Thr Asn Lys Ser Leu Thr Ile His Trp	
620 625 630	
ctg cct cct att agt gga gtt gta tat gac agg gcc tca ggc agc ttg	2640
Leu Pro Pro Ile Ser Gly Val Val Tyr Asp Arg Ala Ser Gly Ser Leu	
635 640 645	
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Cys Gly Ala Cys Thr Glu Asp Gly Thr Phe Arg Gln Tyr Val His Thr	
650 655 660	
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Ala Ser Ser Arg Arg Val Cys Asp Ser Ser Gly Tyr Trp Thr Pro Glu	
665 670 675	
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Glu Ala Val Gly Pro Pro Asp Val Asp Gln Pro Cys Glu Pro Ser Leu	
680 685 690 695	
cag gcc tgg agc cct gag gtc cac ctg tac cac atg aac atg acg gtc	2832
Gln Ala Trp Ser Pro Glu Val His Leu Tyr His Met Asn Met Thr Val	
700 705 710	
ccc tgc ccc aca gaa ggc tgt agc ttg gag ctg ctc ttc caa cac ccg	2880
Pro Cys Pro Thr Glu Gly Cys Ser Leu Glu Leu Leu Phe Gln His Pro	
715 720 725	
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Val Gln Ala Asp Thr Leu Thr Leu Trp Val Thr Ser Phe Phe Met Glu	
730 735 740	
tcc tcg cag gtc ctc ttt gac aca gag atc ttg ctg gaa aac aag gag	2976
Ser Ser Gln Val Leu Phe Asp Thr Glu Ile Leu Glu Asn Lys Glu	
745 750 755	

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Ser	Val	His	Leu	Gly	Pro	Leu	Asp	Thr	Phe	Cys	Asp	Ile	Pro	Leu	Thr	
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atc	aaa	ctg	cac	gtg	gat	ggg	aag	gtg	tcg	ggg	gtg	aaa	gtc	tac	acc	3072
Ile	Lys	Leu	His	Val	Asp	Gly	Lys	Val	Ser	Gly	Val	Lys	Val	Tyr	Thr	
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ttt	gat	gag	agg	ata	gag	att	gat	gca	gca	ctc	ctg	act	tct	cag	ccc	3120
Phe	Asp	Glu	Arg	Ile	Glu	Ile	Asp	Ala	Ala	Leu	Leu	Thr	Ser	Gln	Pro	
			795					800					805			
cac	agt	ccc	ttg	tgc	tct	ggc	tgc	agg	cct	gtg	agg	tac	cag	gtt	ctc	3168
His	Ser	Pro	Leu	Cys	Ser	Gly	Cys	Arg	Pro	Val	Arg	Tyr	Gln	Val	Leu	
		810					815					820				
cgc	gat	ccc	cca	ttt	gcc	agt	ggt	ttg	ccc	gtg	gtg	gtg	aca	cat	tct	3216
Arg	Asp	Pro	Pro	Phe	Ala	Ser	Gly	Leu	Pro	Val	Val	Val	Thr	His	Ser	
	825					830				835						
cac	agg	aag	ttc	acg	gac	gtg	gag	gtc	aca	cct	gga	cag	atg	tat	cag	3264
His	Arg	Lys	Phe	Thr	Asp	Val	Glu	Val	Thr	Pro	Gly	Gln	Met	Tyr	Gln	
840					845					850					855	
tac	caa	gtt	cta	gct	gaa	gct	gga	gga	gaa	ctg	gga	gaa	gct	tcg	cct	3312
Tyr	Gln	Val	Leu	Ala	Glu	Ala	Gly	Gly	Glu	Leu	Gly	Glu	Ala	Ser	Pro	
				860					865					870		
cct	ctg	aac	cac	att	cat	gga	gct	cct	tat	tgt	gga	gat	ggg	aag	gtg	3360
Pro	Leu	Asn	His	Ile	His	Gly	Ala	Pro	Tyr	Cys	Gly	Asp	Gly	Lys	Val	
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Ser	Glu	Arg	Leu	Gly	Glu	Glu	Cys	Asp	Asp	Gly	Asp	Leu	Val	Ser	Gly	
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gat	ggc	tgc	tcc	aag	gtg	tgt	gag	ctg	gag	gaa	ggt	ttc	aac	tgt	gta	3456
Asp	Gly	Cys	Ser	Lys	Val	Cys	Glu	Leu	Glu	Glu	Gly	Phe	Asn	Cys	Val	
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gga	gag	cca	agc	ctt	tgc	tac	atg	tat	gag	gga	gat	ggc	ata	tgt	gaa	3504
Gly	Glu	Pro	Ser	Leu	Cys	Tyr	Met	Tyr	Glu	Gly	Asp	Gly	Ile	Cys	Glu	
920					925					930					935	
cct	ttt	gag	aga	aaa	acc	agc	att	gta	gac	tgt	ggc	atc	tac	act	ccc	3552
Pro	Phe	Glu	Arg	Lys	Thr	Ser	Ile	Val	Asp	Cys	Gly	Ile	Tyr	Thr	Pro	
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aaa	gga	tac	ttg	gat	caa	tgg	gct	acc	cgg	gct	tac	tcc	tct	cat	gaa	3600
Lys	Gly	Tyr	Leu	Asp	Gln	Trp	Ala	Thr	Arg	Ala	Tyr	Ser	Ser	His	Glu	
			955					960					965			
gac	aag	aag	aag	tgt	cct	gtt	tcc	ttg	gta	act	gga	gaa	cct	cat	tcc	3648
Asp	Lys	Lys	Lys	Cys	Pro	Val	Ser	Leu	Val	Thr	Gly	Glu	Pro	His	Ser	
		970					975					980				
cta	att	tgc	aca	tca	tac	cat	cca	gat	tta	ccc	aac	cac	cgt	ccc	cta	3696
Leu	Ile	Cys	Thr	Ser	Tyr	His	Pro	Asp	Leu	Pro	Asn	His	Arg	Pro	Leu	

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agg agt gaa cag cca gaa ggt agc ctg aag aaa gag gat gag gtt tgg Arg Ser Glu Gln Pro Glu Gly Ser Leu Lys Lys Glu Asp Glu Val Trp 1020 1025 1030			3792
ctc aaa gtg tgt ttc aat aga cca gga gag gcc aga gca att ttt att Leu Lys Val Cys Phe Asn Arg Pro Gly Glu Ala Arg Ala Ile Phe Ile 1035 1040 1045			3840
ttt ttg aca act gat ggc cta gtt ccc gga gag cat cag cag ccg aca Phe Leu Thr Thr Asp Gly Leu Val Pro Gly Glu His Gln Gln Pro Thr 1050 1055 1060			3888
gtg act ctc tac ctg acc gat gtc cgt gga agc aac cac tct ctt gga Val Thr Leu Tyr Leu Thr Asp Val Arg Gly Ser Asn His Ser Leu Gly 1065 1070 1075			3936
acc tat gga ctg tca tgc cag cat aat cca ctg att atc aat gtg acc Thr Tyr Gly Leu Ser Cys Gln His Asn Pro Leu Ile Ile Asn Val Thr 1080 1085 1090 1095			3984
cat cac cag aat gtc ctt ttc cac cat acc acc tca gtg ctg ctg aat His His Gln Asn Val Leu Phe His His Thr Thr Ser Val Leu Leu Asn 1100 1105 1110			4032
ttc tca tcc cca cgg gtc ggc atc tca gct gtg gct cta agg aca tcc Phe Ser Ser Pro Arg Val Gly Ile Ser Ala Val Ala Leu Arg Thr Ser 1115 1120 1125			4080
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ggg cag aat cat cag gga cag agc tgt atc cat cgg ccc tgt ggg aag Gly Gln Asn His Gln Gly Gln Ser Cys Ile His Arg Pro Cys Gly Lys 1145 1150 1155			4176
cag gac agc tgt ccg tca ttg ctg ctt gat cat gct gat gtg gtg aac Gln Asp Ser Cys Pro Ser Leu Leu Leu Asp His Ala Asp Val Val Asn 1160 1165 1170 1175			4224
tgt acc tct ata ggc cca ggt ctc atg aag tgt gct atc act tgt caa Cys Thr Ser Ile Gly Pro Gly Leu Met Lys Cys Ala Ile Thr Cys Gln 1180 1185 1190			4272
agg gga ttt gcc ctt cag gcc agc agt ggg cag tac atc agg ccc atg Arg Gly Phe Ala Leu Gln Ala Ser Ser Gly Gln Tyr Ile Arg Pro Met 1195 1200 1205			4320
cag aag gaa att ctg ctc aca tgt tct tct ggg cac tgg gac cag aat Gln Lys Glu Ile Leu Leu Thr Cys Ser Ser Gly His Trp Asp Gln Asn 1210 1215 1220			4368
gtg agc tgc ctt ccc gtg gac tgc ggt gtt ccc gac ccg tct ttg gtg			4416

Val Ser Cys Leu Pro Val Asp Cys Gly Val Pro Asp Pro Ser Leu Val	
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Asn Tyr Ala Asn Phe Ser Cys Ser Glu Gly Thr Lys Phe Leu Lys Arg	
1240 1245 1250 1255	
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Cys Ser Ile Ser Cys Val Pro Pro Ala Lys Leu Gln Gly Leu Ser Pro	
1260 1265 1270	
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Trp Leu Thr Cys Leu Glu Asp Gly Leu Trp Ser Leu Pro Glu Val Tyr	
1275 1280 1285	
tgc aag ttg gag tgt gat gct ccc cct att att ctg aat gcc aac ttg	4608
Cys Lys Leu Glu Cys Asp Ala Pro Pro Ile Ile Leu Asn Ala Asn Leu	
1290 1295 1300	
ctc ctg cct cac tgc ctc cag gac aac cac gac gtg ggc acc atc tgc	4656
Leu Leu Pro His Cys Leu Gln Asp Asn His Asp Val Gly Thr Ile Cys	
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Lys Tyr Glu Cys Lys Pro Gly Tyr Tyr Val Ala Glu Ser Ala Glu Gly	
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Lys Val Arg Asn Lys Leu Leu Lys Ile Gln Cys Leu Glu Gly Gly Ile	
1340 1345 1350	
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Trp Glu Gln Gly Ser Cys Ile Pro Val Val Cys Glu Pro Pro Pro Pro	
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Val Phe Glu Gly Met Tyr Glu Cys Thr Asn Gly Phe Ser Leu Asp Ser	
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cag tgt gtg ctc aac tgt aac cag gaa cgt gaa aag ctt ccc atc ctc	4896
Gln Cys Val Leu Asn Cys Asn Gln Glu Arg Glu Lys Leu Pro Ile Leu	
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tgc act aaa gag ggc ctg tgg acc cag gag ttt aag ttg tgt gag aat	4944
Cys Thr Lys Glu Gly Leu Trp Thr Gln Glu Phe Lys Leu Cys Glu Asn	
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ctg caa gga gaa tgc cca cca ccc ccc tca gag ctg aat tct gtg gag	4992
Leu Gln Gly Glu Cys Pro Pro Pro Pro Ser Glu Leu Asn Ser Val Glu	
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Tyr Lys Cys Glu Gln Gly Tyr Gly Ile Gly Ala Val Cys Ser Pro Leu	
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Cys Val Ile Pro Pro Ser Asp Pro Val Met Leu Pro Glu Asn Ile Thr	
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 Ala Asp Thr Leu Glu His Trp Met Glu Pro Val Lys Val Gln Ser Ile
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 Val Cys Thr Gly Arg Arg Gln Trp His Pro Asp Pro Val Leu Val His
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 1500 1505 1510

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 Ile Asn Asn Arg Ala Tyr Cys His Tyr Asp Gly Gly Asp Cys Cys Ser
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 Ser Thr Leu Ser Ser Lys Lys Val Ile Pro Phe Ala Ala Asp Cys Asp
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 Leu Asp Glu Cys Thr Cys Arg Asp Pro Lys Ala Glu Glu Asn Gln
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 His His Leu Phe Gly Val Tyr Pro Ser Arg Ala Gly Asn Tyr Leu Arg
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 Pro Tyr Pro Val Gly Glu Gln Glu Ile His Thr Gly Arg Ser Lys
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 Pro Asp Thr Glu Gly Asn Ala Val Ser Leu Val Pro Pro Asp Leu Thr
 100 105 110
 Glu Asn Pro Ala Gly Leu Arg Gly Ala Val Glu Glu Pro Ala Ala Pro
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 Trp Val Gly Asp Ser Pro Ile Gly Gln Ser Glu Leu Leu Gly Asp Asp
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 Asp Ala Tyr Leu Gly Asn Gln Arg Ser Lys Glu Ser Leu Gly Glu Ala
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 Gly Ile Gln Lys Gly Ser Ala Met Ala Ala Thr Thr Thr Ala Ile
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 Phe Thr Thr Leu Asn Glu Pro Lys Pro Glu Thr Gln Arg Arg Gly Trp
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 Ala Lys Ser Arg Gln Arg Arg Gln Val Trp Lys Arg Arg Ala Glu Asp
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 Gly Gln Gly Asp Ser Gly Ile Ser Ser His Phe Gln Pro Trp Pro Lys
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 His Ser Leu Lys His Arg Val Lys Lys Ser Pro Glu Glu Ser Asn
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 Gln Asn Gly Gly Glu Gly Ser Tyr Arg Glu Ala Glu Thr Phe Asn Ser
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 Gln Val Gly Leu Pro Ile Leu Tyr Phe Ser Gly Arg Arg Glu Arg Leu
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 Leu Leu Arg Pro Glu Val Leu Ala Glu Ile Pro Arg Glu Ala Phe Thr
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 Ile Ala Gly Val Phe Asp Asn Cys Ser His Thr Val Ser Asp Lys Gly
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Ala	Ser	Cys	Arg	Ser	Leu	Leu	Leu	Gly	Gly	Asp	Ser	Ser	Glu	Asp	Gly
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His	Tyr	Phe	Arg	Gly	His	Leu	Gly	Thr	Leu	Val	Phe	Trp	Ser	Thr	Ala
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Leu	Gln	Gly	Phe	Glu	Pro	Glu	Pro	Glu	Ile	Leu	Ser	Pro	Leu	Gln	Pro
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Pro	Leu	Cys	Gly	Gln	Thr	Val	Cys	Asp	Asn	Val	Glu	Leu	Ile	Ser	Gln
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Glu	Gln	Ile	Arg	Leu	Gln	His	Glu	Ala	Leu	Asn	Glu	Ala	Phe	Ser	Arg
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Tyr	Asn	Ile	Ser	Trp	Gln	Leu	Ser	Val	His	Gln	Val	His	Asn	Ser	Thr
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Gly	Gly	Asp	Cys	Arg	Leu	Gln	Gly	Arg	Cys	Tyr	Ser	Trp	Asn	Arg	Arg
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Asp	Gly	Leu	Cys	His	Val	Glu	Cys	Asn	Asn	Met	Leu	Asn	Asp	Phe	Asp
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Asp	Gly	Asp	Cys	Cys	Asp	Pro	Gln	Val	Ala	Asp	Val	Arg	Lys	Thr	Cys
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Phe	Asp	Pro	Asp	Ser	Pro	Lys	Arg	Ala	Tyr	Met	Ser	Val	Lys	Glu	Leu
			660					665					670		
Lys	Glu	Ala	Leu	Gln	Leu	Asn	Ser	Thr	His	Phe	Leu	Asn	Ile	Tyr	Phe
	675						680					685			
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Asp	Lys	Asp	Ala	Val	Thr	His	Leu	Gly	Gly	Ile	Val	Leu	Ser	Pro	Ala
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Tyr	Tyr	Gly	Met	Pro	Gly	His	Thr	Asp	Thr	Met	Ile	His	Glu	Val	Gly
			725					730						735	
His	Val	Leu	Gly	Leu	Tyr	His	Val	Phe	Lys	Gly	Val	Ser	Glu	Arg	Glu
			740					745					750		
Ser	Cys	Asn	Asp	Pro	Cys	Lys	Glu	Thr	Val	Pro	Ser	Met	Glu	Thr	Gly
		755					760					765			
Asp	Leu	Cys	Ala	Asp	Thr	Ala	Pro	Thr	Pro	Lys	Ser	Glu	Leu	Cys	Arg
	770					775					780				
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785					790					795				800
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			820					825					830	
Val	Tyr	Gln	Gln	Trp	Thr	Glu	Ser	Arg	Lys	Pro	Thr	Pro	Ile	Pro
		835					840					845		Ile
Pro	Pro	Met	Val	Ile	Gly	Gln	Thr	Asn	Lys	Ser	Leu	Thr	Ile	His
	850					855					860			Trp
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865					870					875				Leu
Cys	Gly	Ala	Cys	Thr	Glu	Asp	Gly	Thr	Phe	Arg	Gln	Tyr	Val	His
				885					890					Thr
Ala	Ser	Ser	Arg	Arg	Val	Cys	Asp	Ser	Ser	Gly	Tyr	Trp	Thr	Pro
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Ser	Val	His	Leu	Gly	Pro	Leu	Asp	Thr	Phe	Cys	Asp	Ile	Pro	Leu
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Ile	Lys	Leu	His	Val	Asp	Gly	Lys	Val	Ser	Gly	Val	Lys	Val	Tyr
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Lys	Gly	Tyr	Leu	Asp	Gln	Trp	Ala	Thr	Arg	Ala	Tyr	Ser	Ser	His
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Asp	Lys	Lys	Lys	Cys	Pro	Val	Ser	Leu	Val	Thr	Gly	Glu	Pro	His
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Thr	Gly	Trp	Phe	Pro	Cys	Val	Ala	Ser	Glu	Asn	Glu	Thr	Gln	Asp
	1235					1240					1245			Asp
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 Val Thr Leu Tyr Leu Thr Asp Val Arg Gly Ser Asn His Ser Leu Gly
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 His His Gln Asn Val Leu Phe His His Thr Thr Ser Val Leu Leu Asn
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 Ser Arg Ile Gly Leu Ser Ala Pro Ser Asn Cys Ile Ser Glu Asp Glu
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